Biochemistry and Physiology of Nutrition

VOLUME I

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VOLUME I

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Preface

This treatise was undertaken to gather together into one work the most salient segments of the vast amount of research dealing with the field of nutrition It was written by specialists and is directed to all workers dealing with problems of metabolism as well as to the nutritionist, for the science of nutrition has come to encompass metabolic reactions as well as raw materials

The beginning of the science of nutrition dates from about the middle of the nineteenth century, so that the present work represents the result of one hundred years of development of this subject, a century of nutritional progress In planning this work, we have not forgotten the past The chapters by Cathcart, Harris, and the historical section of that of Goldsmith serve to remind us not only of the small and tentative beginnings of the then new science but of the giants of the past and their contributions to the streams of fact and fancy which, through the century, have joined and broadened to form a great river of knowledge In our approach to the modern aspects of nutrition we have given considerable space to the treatment of enzymes and coenzymes We feel that basic metabolic functions have assumed a definite and indispensable place in the science of nutrition It is no longer adequate to consider only the materials consumed by the organism, we must look as well to the biosynthetic mechanisms if we are to follow through to the final products In this way understanding may be reached, for instance, as to the ultimate proteins which are constructed from the amino acids, whether these amino acids are required in the diet or synthesized *de novo* Also the chemical entities classed as vitamins rarely function as such but must be constructed into specific coenzymes Therefore, this work contains extensive discussions not only of the major nutritional components, carbohydrates, fats, proteins, minerals, vitamins, and water, but also of hydrolytic and phosphorolytic enzymes, respiratory enzymes, and their coenzymes

The sections on the B vitamins and the fat-soluble vitamins have been somewhat condensed because this material has been extensively covered in many recent reviews Certain specific aspects of these compounds and their relation to the organism as a whole, however, have received extensive treatment Such is the case in the chapter on hematopoesis Vitamin C and associated factors has been dealt with at length

Sections are included on certain aspects of histochemistry and the relationship of cellular structures to nutritional state The role of microorganisms in the over-all nutritional picture of animals has been treated, as has also the biochemical aspects of the nutrition of invertebrate animals,

Although the major emphasis is placed on the animal organism, the reader will find innumerable references to bacteria and fungi, especially where enzymatic function is discussed, illustrating the extent to which the field of microbiology has contributed invaluable insight into general metabolic functions

One of the prime functions of a comprehensive treatise is to point up the gaps in our knowledge and to stimulate investigation which will ultimately, bit by bit, eliminate them We are certain that in such a dynamic science as nutrition those gaps will be easily discernible to the reader, and we have the utmost confidence in the ingenuity of future workers in the field for finding more answers

The Editors were aware from the start, in undertaking the construction of the present treatise, that certain phases rightly belonging to "nutrition" would have to be curtailed or omitted Also it was recognized that some repetitions would be inevitable Although authors were given complete freedom in bringing their chapters as nearly up-to-date as possible by extensive revision of galley proof, some of the latest literature does not appear, thus reflecting the unavoidable time lag between proof and final publication

This work is an Anglo-American effort, as a result of which some slight discrepancies of terminology in different sections will be noted by the reader The planning of the work and the selection of the topics and the contributors have been the responsibility of the Editors The thoroughness and originality of expression are those of the individual authors, and all credit goes to them

The Editors wish to express their gratitude to the publishers for making this contribution to science possible and for allowing such freedom for proof revision They also express gratitude to Dr Virginia C. Dewey for her preparation of the subject index

G. H. Bourne
G. W. Kidder

June, 1953

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By T. H. Jukes, *Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York*

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CHAPTER 1

The Early Development of the Science of Nutrition

E. P. CATHCART

Emeritus Professor of Physiology, University of Glasgow

I. Introduction

There probably will be many who will ask what is the use of an introductory chapter of this kind, a chapter which makes no pretense of being up to date, which professes no contribution to any of the current hypotheses, but is simply an attempt to give the readers of this book a brief account of the foundations on which the modern science of nutrition has been built. Ancestor worship, if you like, is perhaps not so exciting as some other studies, but it is well to admit, from time to time, that we owe much to our scientific forebears. Remember that all the commonplaces of our modern textbooks, at some period or other in the past, were exciting new contributions to the science of the time. Let us not forget the wise words of that original worker and thinker Claude Bernard: "We stand upon the intellectual shoulders of the medical giants of bygone days and, because of the help they afford us, we are able to see more clearly than they were able to do."

II. Phlogiston

It is not overstating the fact to say that the keynote of practically all chemistry towards the end of the eighteenth century was dominated by *phlogiston*, a hypothetical substance put forward by the German Stahl and

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introduced into France by Senac Even the best of the scientific brains of the time, like Black and Lavoisier, if they doubted the relevancy of the conclusions to which their work pointed, were afraid publicly to state their disbelief in the reality of this intangible, all-pervasive essence, which proved such a handicap in the elucidation and interpretation of their scientific results And what was this incubus, devised by Stahl, which so hampered the research workers of the time? Briefly the theory was as follows When a combustible body (i e , a phlogisticated body) was burned, something, which Stahl called phlogiston, departed from it; the body suffered loss and became dephlogisticated. No human eye ever saw phlogiston, just as our generation has never seen energy.

1 Lavoisier

It is commonly said that the advent of Lavoisier set a very definite limit to the separation of what may be called the old and the new chemistry If such a view be accepted, consider the list of contemporaries: Lavoisier (1743–1794), Black (1728–1799), Crawford (1748–1795); Priestley (1733–1804), Cavendish (1731–1810), Rutherford (1749–1818), Haller (1708–1777) Lavoisier was *certainly not the only bright star* in the firmament What had these other investigators who were admitted by Lavoisier to be his equals and sometimes his superior done? He held Black and Priestley in particular in the highest esteem Priestley was spoken of thus. "Celui qui devait est le rival glorieux de Lavoisier, l'illustre Priestley"; and Black was hailed thus· "C'est un des plus zeles admirateur de la profondeur de votre genie " It has been said, with a certain amount of justification, that Lavoisier only completed the foundations on which the grand structure of modern chemistry has since arisen Lavoisier certainly derived a great deal of inspiration from the work of Priestley and Black

2 Black

Despite his originality, the work of Black is less well known than that of many other investigators His fundamental studies, looked at today, seem to be almost ridiculously simple, and yet, for the first time, they provided ample material which might have been used to demolish the *phlogiston myth forever Black found that ordinary lime lost in weight* when it was burned to the caustic state, that mild alkalis (lime, magnesium, etc) gave off a particular kind of air when they were treated with acid, and that caustic lime on exposure to the atmospheric air became mild—something therefore had been lost to the atmosphere Quicklime did not attract ordinary air but, as Black wrote, it is "capable of being joined to one particular species only, which is dispersed through the atmosphere, either in the shape of an exceedingly subtle powder or, more probably, in that of

an elastic fluid. To this I have given the name of 'fixed air.'" Black also found that, when mild lime was burned and became caustic lime, 'fixed' air was given off. The loss of weight, when this mild lime was converted into caustic lime by burning, was due to the loss of 'fixed air.' He also noted that precipitation took place when 'fixed air' was passed into lime-water, that in fermentation 'fixed air' was given off, that burning charcoal gave off 'fixed air,' and that it was also found in the expired air from the lungs. Black's 'fixed air' is carbon dioxide. Black also discovered latent and specific heats.

As already remarked, these experiments of Black could have destroyed the phlogiston hypothesis but for the strength of the beliefs then current. I fancy that Black was not a fervent upholder of the phlogiston theory, as he was one of the first to support Lavoisier when he enunciated his theory.

As regards the contribution of Cavendish, he discovered 'inflammable air' (hydrogen) and believed that it was phlogiston. He determined the specific gravity of hydrogen and carbon dioxide. He noted, too, that 'fixed air' derived from fermentation was the same as that derived from marble. He discovered the synthesis of water, that when a mixture of two parts of hydrogen ('inflammable air') and one part of the 'dephlogisticated air' of Priestley (oxygen) were exploded the airs disappeared and water was left. He also carried out a series of tests concerning the uniformity of the composition of the atmosphere and, incidentally, noticed that a very small fraction of the dephlogisticated air of the atmosphere differed from the rest. Cavendish was not so strong a believer in phlogiston as Priestley.

3. PRIESTLEY

Priestley was a truly remarkable man, with an infinite variety of interests. He was by profession a non-conformist minister and had held several charges. At first he devoted his attention to oriental languages, then to logic and metaphysics, then to modern languages. He had a perfect passion for experimental chemistry and physics, but much of his work was incomplete, as he was quite happy carrying out experiments with no ultimate end in view. He had had no scientific training of any kind. The theories he propounded were little more than guesses, but his ability to draw deductions and his capacity for observation were sound. His great discovery was 'dephlogisticated gas' (oxygen), a discovery to which Priestley paid but little attention. It only fell into line with the other gases he had discovered, 'alkaline air' (ammonia), 'marine acid gas' (hydrochloric acid), and 'nitrous air' (nitric acid). Priestley held strong views on many subjects; some were revolutionary, which brought him eventually to grief as his house was attacked by the mob and he had to flee. He emigrated to the United States in 1794 and died in Northumberland, Pennsylvania, in 1804.

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4. Scheele

Scheele, a poor Swedish apothecary, discovered oxygen before Priestley, but the fact had never been properly published. Scheele found that, if he exposed silver carbonate to heat and collected the gas which was set free, he obtained a mixture of gases. There was the 'fixed air' of Black, which he removed by passing the gases through lime water; this left him with a new gas which he called 'pure air.' He found that a flame lit in this gas burned very brightly. He carried out another extraordinary neat, interesting, and suggestive experiment in which he placed the lower end of a small cylinder previously filled with 'pure air' in limewater so that the carbon dioxide might be absorbed as it was formed by some bees which he had placed in a little chamber at the top end of the cylinder. As the result of the respiration of the bees, the air volume in the cylinder slowly diminished, and eventually, after 8 days, the bees were dead. It is almost certain that the 'air' he used for filling the cylinder was 'pure air' (oxygen).

III. Combustion and Respiration

I think it may be definitely affirmed that Lavoisier's interest up till about 1777 might be summed up in one word—*combustion*. He was intensely interested in what we now call oxidation and reduction. In 1777 he was dissatisfied with the current explanations of certain chemical events. At the Easter Meeting of the Academy of Sciences in that year Lavoisier read a paper which proved to be the first serious attack on the phlogiston theory. This memorable memoir was entitled "The Nature of the Principle Which Combines with Metals during Their Calcination and Which Increases Their Weight." In this paper he detailed experiments on the reduction of mercury calx by charcoal and by heat alone. The air or gas given off during the tests with heat alone, was found to be new. It was not soluble in water, there was no precipitation when it passed through limewater, it did not unite with fixed alkalis, and flames burned more brightly in it. Just previous to these experiments of Lavoisier, Priestley had discovered what he called 'dephlogisticated air' which he had obtained by heating mercuric oxide. Lavoisier, when he heard of the discovery, grasped the fact that Priestley's 'dephlogisticated air' was the principle he was looking for. Lavoisier called this dephlogisticated air 'an eminently respirable' or 'oxygine principle.'

There was no mention made in Lavoisier's paper of the work of Priestley, although it was Priestley's random discovery that gave Lavoisier the clue. Priestley undoubtedly discovered oxygen, as he did other gases, but he did not appreciate the importance of this new gas. Lavoisier, later, probably under pressure, gave a very paltry excuse for the omission of Priestley's

name—"The air which Mr Priestley discovered at very nearly the same time as I, and I believe even before me . "

Lavoisier, having satisfied himself about the chemistry of oxidation and reduction, turned to the investigation of respiration, turned, so to speak, from the study of inorganic chemistry to biological chemistry

Again Lavoisier was attracted to the work of Priestley, who had shown that, if air in a closed vessel was rendered 'noxious,' either by burning a candle in it or by keeping a small bird or mouse in it, the vitiated air could be made once more respirable if a small amount of green vegetation were introduced into it Lavoisier published another paper in 1777 on "Experiments upon the Respiration of Animal and upon the Changes Which the Air Undergoes in Passing through the Lungs" Here it was shown that burning and respiration were equally effective in giving rise to noxious air

Lavoisier began a series of experiments to substantiate these statements There proved, however, to be an essential difference between air in which a metal had been calcined and air which had been breathed The air which had been breathed contained Black's 'fixed air,' for it caused a precipitation when passed through limewater Lavoisier did not doubt that the 'fixed air' came from the lungs and therefore recognized that two changes came into operation in respiration (1) the disappearance of oxygen and (2) the appearance of fixed air In one of his earliest experiments, carried out to elucidate the problem, he put a mouse into a bell jar full of air and sealed with mercury The small animal died in 55 minutes, and Lavoisier noted that there was a decrease of about one-sixth in the volume of the air in the jar The air that remained, when tested with limewater, gave a precipitation, and it extinguished a flame exposed to it A bird introduced into the air remaining in the jar died almost at once

Lavoisier did not like the term 'fixed air,' he preferred to call it 'aeriform acid' or 'calcic acid ' Hence air vitiated by breathing contains one-sixth part of an 'aeriform acid' like that which was obtained from chalk When the 'fixed air' was removed, the air left was, to all intents and purposes, like the air in which a metal had been calcined This air could extinguish a flame and was unfit for breathing As this air could not support life, Lavoisier called it 'azotic acid' or 'azote ' When this air, in its turn, was mixed with one-fourth of its volume of 'dephlogisticated air,' (oxygen) it became identical with common or atmospheric air.

Lavoisier came to the conclusion that in respiration there was utilized only the 'eminently respirable air,' the remainder of the atmosphere, the mephitic part or azote, was left unchanged Of the ordinary air we breathe, he maintained that five-sixths was in the mephitic state and therefore could not be utilized for ordinary respiratory purposes He noted, too, that animals which were confined to a limited space died as soon as they

had consumed the greater part of the respirable fraction of the atmosphere, converting it into 'fixed air' or 'aeriform calcic acid.' He further observed that in the case of metals calcination in atmospheric air proceeded only until the 'eminently respirable air' (or oxygen) was used up.

Lavoisier, in association with the mathematician Laplace, published their classical memoir on heat, using an ice calorimeter, modified from one of Black's They measured the relative quantity of heat freed in the course of chemical reactions by measuring the amount of ice which was melted with each test They demonstrated that respiration was, in essence, a very slow combustion, similar to that of charcoal. This slow combustion was, in their opinion, the sole cause of animal heat. In measuring the amount of carbon dioxide produced in respiration and comparing this with the quantity of heat yielded, Lavoisier soon established the fact that the quantity of heat liberated was greater than that which would have been furnished by the amount of carbon consumed. He concluded that respiration is not limited to the combustion of carbon (charcoal) alone In 1785 he found, from experiment, that there was also a utilization of the hydrogen contained in the blood, the hydrogen which can combine with 'vital air' to form water

With the aid of Sequin, Lavoisier studied the phenomenon of transpiration and showed that this was the regulator of animal heat Not content with determining the conditions of the act of respiration in the state of rest, he measured the amount of heat liberated during work and discovered that muscular work increased the respiratory combustion and that this in turn brought about an increase of heat. He established irrefutably the relationships which exist among work, heat, respiration, transpiration, and digestion, which in turn give back to the machine all that it loses by transpiration and respiration As Grimaux has stated, "for the first time the vital activities were related to physico-chemical activity and the transformation of work into heat was established in the phenomenon of respiration."

Black, many years before this time, by his studies on heat (it must be remembered that at this period heat was looked upon as something tangible, as an entity or substance which combined with the thing heated) differentiated between latent and sensible heat and put forward his views on capacity for heat and of specific heat Crawford, one of Black's pupils, published a book in which he expressed a theory based on Black's work The results he obtained were admirable and most interesting; the difficulty lies in the deductions he drew. It has been suggested that he anticipated Lavoisier in his appreciation of chemical respiration. Whether or not he did is of little importance, for much of Crawford's theory is quite untenable. For example: "Transpired air contains elementary fire and in the lungs it meets with the inflammable principle which is present in the blood This elementary fire

in turn leaves the air of the lungs to join the blood, the capacity of which for heat is increased" But Crawford was not foolish in the eyes of his generation, in the deductions he drew from his research work He also wrote this: "In the same animal the degree of heat is in some measure proportional to the quantity of air inspired in a given time Thus we find that animal heat is increased by exercise and whatever accelerates respiration"

Lavoisier supposed that the oxidation of the carbon and hydrogen ingested with food took place in the tissue of the lungs, as the oxidation of a 'hydrocarbonous fluid' secreted into the tubes of the lungs. Lagrange the mathematician, however, pointed out that, if all the heat distributed throughout the body were set free in the lungs, the temperature of the lungs would, as a result, be so elevated as to bring about their destruction. He therefore concluded that it was possible that the liberation of heat took place, not in the lungs alone, but in the body at large, where there was a circulation of blood He held the good modern view that the blood in passing through the lungs takes up oxygen from the inspired air and that this oxygen-rich blood is carried away into the circulatory system and slowly gives up its oxygen to combine in part with the carbon and in part with the hydrogen of the blood and thus to form carbonic acid and water, which are set free from the venous blood, as soon as this leaves the right side of the heart to enter the lungs

Although the evidence against the existence of phlogiston was steadily increasing, even at a late date (1789) Lavoisier hesitated about attacking it openly There are three letters from Lavoisier to Black which lend support to this hesitation

In the first letter, dated the 19th day of September, 1789, Lavoisier writes. "It is as one of your keenest admirers of the greatness of your genius, and of the important revolutions that your discoveries have brought about in science . " Then, referring to his antiphlogiston views he gives Black a summary of his own work "You will find the development of a new doctrine, which I believe, is more simple and in greater agreement with the facts than that of phlogiston" Then, in a second letter of July 24th, 1790, he thanks Black for the support he has given (neither Priestley nor Cavendish would at this time accept Lavoisier's views): "Your approval, Sir, gets rid of my doubts and gives me more courage" In the third letter, dated November 19th, 1790, Lavoisier gives a most interesting account of his various experiments in relation to respiration "M T. has been a witness during his stay in Paris of some experiments that I have made on respiration in which he wished to take a part We have established the following facts.

"1 The quantity of vital air, or oxygen, that a man in repose and fasting consumed or rather converts into fixed air or carbonic acid dur-

ing one hour is approximately 1200 cubic pouces of France, when he is in an environmental temperature of 26 degrees. [Lusk states that one cubic pouce equals 0.0198 liters.]

"2 The quantity rises to 1400 cubic pousse under the same conditions, if the individual is in an environmental temperature of 12 degrees only.

"3 The quantity of oxygen consumed or converted into carbon dioxide is augmented during the duration of digestion and is raised to 1800–1900 cubic pousses.

"4 By movement and exercise it is carried up to 4000 cubic pousses or even more.

"5 The animal heat remains constant in all cases.

"6 Animals can live in vital air or oxygen, which is not renewed as long as one likes, provided care has been taken to absorb the carbon dioxide as it develops by caustic alkali so that this gas, contrary to what one believed, does not need to be mixed with a certain amount of nitrogen, to render it fit for breathing.

"7 Animals do not appear to suffer in a mixture of fifteen parts of nitrogen gas and one part of oxygen, provided one has taken the precuation to absorb the carbon dioxide, by means of caustic alkali, as quickly as it is formed.

"8 The consumption of oxygen and its conversion into carbon dioxide is the same in pure oxygen and in oxygen mixed with nitrogen, so that respiration is in no way accelerated on account of the purity of the air.

"9. Animals can live quite a long time in a mixture of two parts of hydrogen and one part of oxygen.

"10 Nitrogen gas plays no part in the respiratory act and is expired from the lungs, the same in quantity or quality, as it was inspired.

"11 When, by exercise and movement, there is an increased consumption of oxygen in the lung, circulation accelerates which is easily proved by the beat of the pulse and, in general, when the individual breathes easily. The quantity of oxygen is proportional to the increase in the number of pulsations multiplied by the number of inspirations.

"It is only right, Sir, that you should be one of the first to be informed of the progress that has been made in a career that you have opened up and in which we regard ourselves as your disciples."

With these letters, which sum up Lavoisier's work on respiration, I close this brief review of only one side of his active research. How Lavoisier managed to do the work he did, in the amount of time at his disposal, with apparently no assistance from 'ghosts' is astonishing. It is indeed amazing that Lavoisier ever found time to do any consecutive research work. The

Academy of Sciences, to which he had been elected at the early age of 25 alone made great demands on his time by asking for reports on all sorts of questions He was a member of the Gunpowder Committee and took a keen interest in its proceedings, he was a Collector of Taxes (a Fermier Générale), he ran an experimental farm, he was a Member of the Provincial Assembly of Orleans, he carried on a large correspondence and was well read (or his wife was well read) He owed much to the active help of his wife The only time he could give to his own research was 6 to 9 A M. and 7 to 10 P M He hoped to be able to get one day a week for his own work Of course, in addition to what might be called his biological interests, he was interested in and reported on other phases of chemical activity He laid great stress on the need for accurate instruments, if good work was to be done He had one balance which was accurate to one-tenth of a milligram—very good for the time

IV. Animal Heat

Following Lavoisier and building on the foundations he had laid were many continental investigators, at first mainly French The Academy of Sciences, in order to stimulate research work of a biological nature, created a prize This prize was won by Depretz (1792–1863), with Dulong (1785–1838) as runner-up Depretz investigated once more the problem of respiration and animal heat He came to the conclusion that the only thing that mattered in the genesis of heat was respiration Other suggested factors like friction were of negligible importance He also suggested that in warm-blooded animals there was some arrangement which allowed them to develop the heat which in turn keeps the animal temperature about 37° above the melting point of ice He thought furthermore that possibly some of the oxygen inspired was used for the formation of water Dulong also [illegible] lorimeter [illegible] study of [illegible] stuffs was the sole source of nitrogen for the building up of the nitrogen-containing body tissues Nitrogen-free foodstuffs are not capable of being formed into nitrogen-containing foodstuffs He maintained that nitrogen was essential for life

V. Intake and Excretion of Nutrients

Boussingault (1802–1884) was one of the first to investigate the relation of the carbon, hydrogen, nitrogen and oxygen contents of the maintenance diet of a cow in milk, with the same elements in the excreta (urine, feces, and milk) He was one of the founders of agricultural chemistry and broke much new ground He again reaffirmed the importance of nitrogen in the diet With Le Bel he made the first complete analysis of cows' milk.

He believed that the nature of the fodder did not influence the quantity and chemical properties of milk, provided that the cow received the same relative nutritive equivalent in the fodder. Barral (1819–1884) was the first to employ Boussingault's technique to experiments on human beings. The subject of animal heat was still a problem of continuing interest. Indeed one might venture to say it was the commencement of the era of calorimeters Regnault (1810–1878), in cooperation with Reiset, devoted his attention to the respiration of animals, using a special chamber method. In this device the carbon dioxide produced could be collected and the oxygen measured as it was admitted to the chamber to make up the loss of volume. Although they agreed fundamentally with Lavoisier that animal heat was derived from the simple oxidation of carbon and hydrogen, they doubted whether the problem could be dismissed so simply and so easily They rightly maintained that oxidation was a very complex process, with many intermediate stages, a belief that was shared by Liebig.

VI. Biochemistry and Liebig

We now turn to the development of biochemistry in Germany, where the research was along another line, with investigators like Liebig, Pettenkofer, Voit, Pfluger, and Rubner, who laid down new lines of attack, lines that continue to attract workers today The emphasis at first was on metabolism with special reference to agriculture, later extended to human beings Liebig (1803–1873) was the best known of these workers It was not until 1824 when he was appointed Professor in the University of Giessen that he established a 'school' whose activity was far reaching Liebig was a masterly investigator, he did not confine his attention to any single line of research In conjunction with his lifelong friend and co-worker, Wöhler, he founded organic chemistry, the methods of analysis of organic compounds, the application of chemistry to biological problems, mainly agricultural and work on gases Liebig really began metabolic studies, both respiratory and urinary (nitrogenous). He stated, in addition, that the quantity of food necessary in both conditions must vary in the same ratio Moreover, he was quite definite that the source of animal heat was due to the interaction between the elements of the food and oxygen. Liebig compared the animal body to a furnace which we supply with fuel He held that "it signified nothing what intermediate forms food may assume, what changes it may undergo in the body, the last change is uniformly the conversion of its carbon into carbonic acid and of its hydrogen into water. The unassimilated nitrogen of the food, along with the unburned or unoxidized carbon, is expelled in the urine or in the solid excrements In order to maintain the furnace at constant temperature we must vary the supply of fuel according to the external temperature, that is according to the supply of oxygen"

Liebig laid great stress on the established fact that the animal body is "absolutely incapable of producing or creating an elementary body such as sulfur, carbon or nitrogen out of substances, which do not contain them." He was very definitely of the opinion that the training of the investigator should be broad. "Numberless examples prove that the wisest and most acute of men have regarded as impossible, facts and suppositions, because they were incomprehensible to their understandings, while their posterity have not only found these things comprehensible but, further, everyone now knows them as established incontrovertible truths." "It is only in regard to the causes, by which the effect is produced, that doubts can exist. These causes may be quite unknown but they can never, in the province of natural science, be ascertained by the power of imagination." Liebig put forward a classification of the various food-stuffs. The group of materials containing nitrogen, which serve for the building up of the body tissues, he called *plastic substances*. The other group, which he referred to as *respiratory substances*, are the non-nitrogenous materials—fats and carbohydrates.

I am afraid modern biochemists would learn with astonishment that Liebig believed that "a healthy man is guided by an instinct which prescribes for him the best proportion in which to mix the plastic and non-nitrogenous materials for his diet." Can we be sure that he is wholly wrong? As regards the nitrogenous materials Liebig was the earliest to appreciate their importance in the dietary, he said: "In no case can life be for long maintained if the proportion of the nitrogenous constituents be reduced below a certain fixed minimum." He also pointed out that mineral matter like sodium, potassium, calcium, and so on, is as essential for the life of an animal as the organic matter and it must therefore be provided in the food, at the same time he stated that these mineral substances, although vital to life, cannot serve as sources of energy.

Liebig was influenced in many of his conceptions concerning the nature of the tissues by a substance called *protein* by its discoverer, Mulder. This substance turned out to be an artifact, but, although the substance was discarded, the name was retained as a generic name for large numbers of nitrogen-containing foodstuffs. Other German workers who played an active role in the development of the study of metabolism were Bidder (1810–1894) and Schmidt (1822–1853?), who defined the condition we now call basal metabolism. They stated that there is a typical minimal essential metabolism for every species of animal, which can be determined in experiments, where no food is given, that is, in modern terminology, during the post-absorptive state. They termed any excess intake, over and above that of the basal requirement, luxus consumption. It must be noted, however, that the well-being and the functional activities of the organism are definitely increased as the result of the increased metabolism. They were the

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[illegible]

supply of oxygen."

ant stimulating conversation Voit deprecated especially "Tischgesprache von Medizinern" as a concomitant of a meal

Rubner (1854–1932) was Voit's most distinguished student and assistant He was the first to prove that the conservation of energy was applicable to the animal organism and the first, also, to develop direct calorimetry, incidentally, he built a very accurate calorimeter He developed, for the purpose of accurate standardization of animals of different types and sizes, the surface area formula put foward by Meeh This formula is of particular interest in relation to body weight He played a very large part in the development of the idea that the regulation of the body temperature was one of the fundamental activities of metabolism He pointed out that in ordinary conditions of life for an animal at rest, in a temperate climate, and receiving a maintenance ration the need of heat is, without doubt, the predominant need. Rubner also devised the expedient of isodynamic weight and of specific dynamic action In the first of these he points out that, for example, two maintenance diets are not necessarily isodynamic and, on the other hand, two isodynamic diets are not necessarily physiological equivalents As regards the specific dynamic action Rubner found that after the ingestion of meat in excess of the starvation metabolic needs the so-called specific dynamic action brought about a heat increase of approximately 30% Rubner was also interested in the nature and constitution of the tissue fluids and drew up a most elaborate classification Whereas Voit and Pfluger were each content with two, Rubner postulated the existence of four He based his classification, not on their chemical constitution, but on the degree of stability of their metabolism They are as follows

1 *Organized protein (Organ Eiweiss)* This is a protein retained to cover the needs of protein in growth and in convalescence This was meant to include retention of organized living material

2 *Improvement protein (Melioration Eiweiss)* A normal adult, on a high-protein diet, may retain a certain amount of nitrogen which remains unmetabolized, when the diet is changed to a nitrogen-free one

3 *Transitional protein (Ubergangs Eiweiss)* This is a material which undergoes slow metabolism over a period of two to three weeks of a nitrogen-free diet, in contrast to the improvement protein, which was more stable

4 *Reserve protein (Vorrats Eiweiss)* This appears to be the "circulation" protein of Voit The amount of this material retained at any one time is dependent on the protein content of the diet

Pfluger (1829–1910) may be regarded as the founder of experimental biology Despite his irascible temper, he carried out good work. He devoted a considerable amount of time to the study of the relationship between glycogen and protein He also broke new ground with his study of the gases of the blood He was an excellent designer of scientific apparaus

first investigators to try to determine the total metabolism, respiratory and nitrogenous, following the technique of Boussingault for animal metabolism. They also investigated the respiratory (CO_2/O_2) quotient of various animals. They defined a typical food minimum as the quantity of food, capable of being assimilated, which is required to maintain body weight over a long period of time.

VII. Voit, Rubner, and Metabolism

Carl von Voit (1831–1908) made the biggest contribution, in the early days, to the study of metabolism. He attacked the various metabolic problems from both aspects, the respiratory and the nitrogenous. The respiratory chamber that he built with Pettenkofer (1818–1901), the founder of modern hygiene, was sufficiently large to allow a man to sleep, work, and eat within it. The ingoing and outgoing air and water vapor were constantly sampled and analyzed, and the chamber was tested for tightness with the aid of a burning candle. This was probably the first large chamber to be tested in this way. Voit was also keenly interested in the constitution of the tissue proteins after absorption. The protein ingested became what he called *circulating protein*. This protein was catabolized by the living tissues, without, in his opinion, first becoming an integral of part of them. He believed that the circulating protein is more easily broken down than the tissue protein. From time to time a certain portion of the tissue protein undergoes degeneration and is excreted. This loss may be made good from the circulating protein. Voit and his views were most virulently attacked by Pfluger of Bonn, who believed that the protein of the food became an integral part of the living protoplasm before it could be utilized. It is related that Voit became tired of the constant fight with Pfluger and suggested that the fight should end. All he got in reply was: "The unvarnished truth of my exactly critical reply has seized Voit so that he was thrown into a paroxysm of raving passion and, setting aside a real answer, he has poured on me the most insulting invective." Thus did they conduct their polemics.

When we turn to the dietary studies, the conclusions reached by Voit are very sharply defined. He laid down four criteria for drawing up a sound dietary: (1) every essential food-stuff must be present in sufficient amount, (2) the individual foodstuffs must be present in proper proportion, (3) the materials of the diet must be absorbed from the intestinal canal, and finally Voit becomes positively lyrical on the subject of the fourth essential, that the diet must be tasty, an essential that is only too often, especially in animal experiments, forgotten or ignored today. He claimed that for human beings the food itself should be appetizing, the environmental arrangements pleasant, e.g. clean table cloths, decorations for the table, and pleas-

and 1073 examinations of urea He himself acted as subject He realized that not all the urea came from ingested food He also maintained that, when the bulk of the tissue (protoplasmic mass) is kept uniform, urea represents nearly all the nitrogen which has entered the body from the food When the bulk of the tissues is increased, the urea represents the nitrogen of the food, minus the nitrogen gained by the tissues, and, when the bulk is lessened, the urea represents the nitrogen supplied by the food, plus the nitrogen lost by the tissues The bulk of the body regulates the emission of urea, but the supply of food and the activity of the vital actions regulates its production

There are two other investigators who may be mentioned W Proust was one of the first to develop the ultimate analysis of foodstuffs, using a most interesting piece of apparatus which he devised for the purpose of his experiments The other is Lyon Playfair (later Lord Playfair, 1818–1898), whose work, comes nearest to modern work in nutrition He certainly published for the time a most interesting paper, "The Food of Man in Relation to His Useful Work" He accepted Liebig's classification of food stuffs, except that he believed that plastic substances may contribute some of the energy requisite for tissue activity He expanded the conception of the division of work performed in the body into five divisions: (1) mental, (2) calorific, (3) internal dynamical, (4) external dynamical, and (5) digestive or assimilative His aim was to draw up a balance sheet of the amount of food required for complete health during rest and during work. He defined a full day's work as the equivalent of a daily walk, except on Sunday, of 20 miles performed continuously He gave a list of work carried out in different forms of labor, in foot-tons He also gave rather an interesting account of the army rations of several nations and a list of the diets given to laborers engaged on very hard labor

So far reference for the most part has been made to special work associated with given individuals In the earliest periods the research was concentrated on respiration, a function in which nitrogen plays a very small active part, whereas, in the later work cited, the presence or absence of nitrogen is found to play a dominant role There are, as might have been expected, many phases of metabolism on which many attacks have been made by many investigators and three of these are cited: Retention of Protein, Respiratory Quotient and Protein Minimum, which are all in the opinion of the writer of fundamental importance

I have attempted, in this chapter, to give some account of the research work carried out over the past hundred years This attempt has not been completely achieved because, from time to time, in order to make these earlier researches intelligible, it is difficult to refrain from some reference to what is probable in the future I hope, however, that the result does

VIII. Lawes and Gilbert and Their Successors

So far the account of the various investigations, which form much of the basic data for modern nutritional research, has been of French or German origin, except of course for the early work of Black, Priestley, Cavendish, and others Apart from the great work of Lawes and Gilbert at the famous research station at Rothamsted there is but a limited amount of research to be recorded in Great Britain and none of standing at this early period in America The Americans were too busy at the end of the eighteenth century with their internal affairs, political and otherwise, to worry about such remote things as research They have certainly made up for lost time, they now dominate the nutritional research work of the world, both theoretical and applied

With reference to the agricultural work of Lawes (1843-1900) and Gilbert (1817-1901), Lawes was the practical farmer and Gilbert the research chemist It can be said without question that this work at Rothamsted has exerted a profound influence on the agricultural practice of the world Gilbert, the chemist, was a man of immense perseverance, with an intuitive mind which could forecast the possible results of their experiments He was a pupil of Liebig at the University of Giessen. The outstanding chemical work of these two pioneers was to draw the attention of the agricultural world to the important part played by nitrogen They showed clearly the influential part which nitrogen played in the production of non-nitrogenous bodies, carbohydrates—starch and cellulose in cereals, starch in potatoes, and sugar in root crops—and how nitrogen influenced the maturing of root crops They also showed how fixed bases like potash affected the formation of sugar in root crops and also the function of potash in the carriage of nitric acid They showed further that such bases are not mutually replaceable and that the dominance of one or the other can affect the product.

One of the lesser lights in Britain was E Smith (1818-1874), who published several sound papers on respiration He carried out 'experimental inquiries' into chemical and other phenomena of respiration and their modifications by various physical agencies His technique was new and admirable, as he developed the use of half-face masks with inspiratory and expiratory valves His design of apparatus for collecting carbon dioxide was particularly sound He estimated the amount of carbon dioxide given off during rest and work, with and without food Foods, should, in his opinion, be divided into two classes, viz , those that excite certain respiratory changes, and those that do not Exciting respiratory foods are those containing nitrogen; the non-exciting foods contain fats and carbohydrates

Smith also tackled the question of the output of urea and urinary water, he stated that he carried out 1633 examinations of the excretion of water

CHAPTER 2

The History of Vitamins*

LESLIE J HARRIS

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I. The Discovery of Vitamins

During the nineteenth century, scientists learned how to analyze foodstuffs chemically and ascertained that they were composed of three main types of organic compounds, namely, proteins, carbohydrates, and fats, together with certain mineral elements and water. These components together were found to account for nearly 100% of the chemical analysis, and it was, therefore, natural to assume that they were all that mattered. At the beginning of the twentieth century it was indeed customary to express the nutritive value of a food by its composition in terms of these substances.

* This chapter is based by permission on articles contributed by the author to the *British Encyclopaedia of Medical Practice*, and on portions of his recent book, Vitamins: A Digest of Current Knowledge published by Churchill, London, 1951

fulfil the end desired, that is, to give a picture that will arouse interest in the foundations on which the modern science of nutrition has been built.

For further information on the life history of Lavoisier, see "Lavoisier," by E Grimaux, published by Alcan, Paris, 1888, and "Lavoisier," by J. A. Cochrane, published by Constable and Co, London, 1931

(1) *Empirical Cure of Dietary Deficiency Diseases* Beginning from the xteenth century, it was found little by little that certain diseases, notably urvy, beriberi, and rickets, could be prevented empirically, or cured, by ie inclusion in the diet of small quantities of antiscorbutic, antiberiberi, antirachitic foodstuffs or extracts

(2) *Discovery of Experimental Avitaminosis* In 1890 and 1907, respec-vely, means were found of producing two such deficiency diseases, beriberi id scurvy, experimentally in animals, and hence of making scientifically ntrolled studies of their cause and prevention

(3) *The Concept of Vitamins* Out of these early observations, little by tle grew the idea that these diseases, scurvy, rickets, and beriberi, could regarded as dietary deficiency diseases, caused by the absence from the :t of certain specific protective substances, or vitamins

(4) *Inadequacy of Purified 'Basal Diets'* Contemporaneously with the velopment of the ideas referred to in the two preceding paragraphs, owledge developed to show that a purified basal diet (that is, one con-ning only isolated fats, proteins, carbohydrates, mineral salts, and ter—the principal ingredients of food) was in itself insufficient for main-iance of (*a*) growth, and indeed (*b*) life itself, but that the addition of itable small supplements derived from natural foods could convert this idequate basal diet into one adequate for (*a*) growth and (*b*) normal alth

(5) *Multiplicity of the Vitamins* There then followed the recognition that ore than one substance in such supplements was responsible for the ects produced

(6) *Identity of the Accessory Factors with Vitamins* Next it was established it the 'growth substances' or 'accessory factors' needed to supplement :h synthetic diets were the same as the vitamins which protected against specific deficiency diseases

(7) *The Discovery of Additional New Vitamins* From about 1915 onward, w vitamins became recognized, one after another, and distinguished m those already known—first, vitamins A and B, then vitamin C; then then E, afterwards, what had been called vitamin B was found to be a mplex of several different vitamins, and so on

(8) *Chemical, Biological, and Medical Studies on Vitamins* Also, starting m about the time of the first World War, the discovery of each new amin was the signal for intensive studies aimed at its isolation, at the termination of its structure, at its synthesis, and at a detailed under-nding of its mode of action in the body, of the pathological effects of a ficiency, of its clinical uses, and so on

We may now proceed to discuss these various stages in the history of amin discovery in a little more detail

Then, during the course of the first quarter of the twentieth century—following researches by Lunin[1] in Switzerland, Eijkman[2] in Java, Hopkins[3] in England, and others, to be detailed below—it gradually became apparent that there were in foods other things that mattered nutritionally, as well as these well-recognized main components. These newly detected ingredients, present in foods in extremely small amounts, but nevertheless essential for health, came to be known as vitamins. The term 'vitamine' (originally with a terminal -e) was first used by Funk[4] in 1912. At that time no vitamin had yet been isolated, and their chemical nature was quite unknown. Little by little, one vitamin after another became recognized, each responsible for preventing some different disorder in man or animal. Later, one by one they were to be separated in a pure state, their chemical nature established, and eventually their synthesis accomplished in the laboratory.

It will be the object of this chapter to give an historical account of this discovery of the vitamins, and their differentiation from one another. For clarity, it will be best, however, first to give a definition of what we now understand a vitamin to be.

II. Definition

Vitamins, then, may be defined as substances that (*a*) are distributed in foodstuffs in relatively minute quantities, that (*b*) are distinct from the main components of food (i.e., proteins, carbohydrates, fats, mineral salts, and water), that (*c*) are needed for the normal nutrition of the animal organism, and (*d*) the absence of any one of which causes a corresponding specific deficiency disease.

Examples of the best-known vitamins are vitamins B_1, C, and D. Vitamin D is the factor which protects against rickets, a common ailment of childhood. Vitamin C prevents scurvy, a disorder formerly prevalent among sailors, due to insufficient use of fresh fruits and vegetables. Vitamin B_1 deficiency is the cause of beriberi, a disease widespread in rice-eating countries in the East.

Today, something like twenty vitamins in all are known, but only a few of them have yet been demonstrated to be of practical importance in clinical medicine.

III. The Several Stages in the History of Vitamin Discovery

In the gradual development of knowledge about these dietary factors which we now call vitamins, a number of separate stages may be discerned, as follows:

[1] N. Lunin, *Hoppe Seyler's Z. Physiol. Chemie* 5, 31 (1881).
[2] C. Eijkman, *Geneesk. T*
[3] F. G. Hopkins, *J. Physi*
[4] C. Funk, *J. State Med.*

to 1897 he carried out the earliest work on the extraction of the antineuritic substance (now called vitamin B_1), which he found to be present in the bran of rice but not in 'polished' (milled) rice But the first to state clearly that beriberi was due solely and simply to a dietary deficiency and was not caused by any positive agent or toxin was Eikjman's collaborator, Grijns [15]

Experimental Scurvy In 1907, Holst and Frolich[16] in Christiania (now Oslo) discovered experimental scurvy in guinea pigs With Eikjman's pioneer work on beriberi in mind they rightly considered this to be likewise a deficiency disease and set out to examine the properties of the antiscorbutic substance (now vitamin C)

VI. The 'Vitamine' Theory

Gradually the modern concept of vitamins took form By 1906, Hopkins[17] could refer to scurvy and rickets as "diseases in which for long years we have had knowledge of a dietetic factor" He realized, moreover, that the errors in the diet "although still obscure" were "certainly of the kind which comprises the minimal qualitative factors"

In 1912 Funk,[4] then working on the antiberiberi factor propounded his 'vitamine' theory—i e, he postulated the existence of separate antiberiberi, antiscurvy, antirickets, and antipellagra vitamines [18]

VII. Experiments on Synthetic Diets

In the meantime, experiments had been in progress attacking the problem from an entirely different angle—investigating not deficiency diseases as such, but determining what constituted a physiologically complete diet

Lunin,[1] a pupil of the Swiss biochemist Bunge, first showed in 1881 that animals failed to thrive when kept on an artificial regimen composed of the then known constituents of food, i e, re-purified fat, protein, carbohydrate, mineral salts, and water He concluded that "a natural food such as milk must therefore contain besides these known principal ingredients small quantities of other and unknown substances essential to life"

Similar conclusions were reached by various other workers, of whom the most notable were Socin[19] and Stepp,[20] mention must also be made of Coppola,[21] Hall,[22] Hausermann,[23] Henriques and Hansen,[24] Falta and

[16] G Grijns, *Geneesk Tijdschr Nederland-Indië* 41, 3 (1901)
[16] A Holst and T Frölich, *J Hyg* 7, 634 (1907)
[17] F G Hopkins, *Analyst* 31, 395 (1906)
[18] Then spelt with a terminal -e to indicate their presumed basic nature.
[19] C A Socin, *Hoppe-Seyler's Z physiol Chemie* 15, 93 (1891)
[20] W Stepp, *Biochem Z* 22, 452 (1909)
[21] F Coppola, *Rend accad nazl Lincei*, 6 (i), 362 (1890).
[22] W S Hall, *Arch Anat u Physiol Physiol Abt* 142 (1896)
[23] E Häusermann, *Hoppe-Seyler's Z physiol Chemie* 23, 555 (1897).
[24] V Henriques and C Hansen, *Hoppe Seyler's Z physiol. Chemie* 43, 417 (1905)

IV. Empirical Cures of Deficiency Diseases

Scurvy We have to go back to the sixteenth century for what appear to be the first records of the cure of scurvy—that old scourge of mariners—by such agents as a decoction of spruce needles[5] or oranges and lemons.[6] It was early in the seventeenth century (1601) that Sir James Lancaster[7] introduced the regular use of oranges and lemons into the ships of the East India Company as a preventive against the disease Many others, during the seventeenth and eighteenth centuries, repeatedly confirmed the fact that fresh fruits and vegetables were effective in curing or preventing scurvy [8-11] In 1804 the daily consumption of lemon juice was made compulsory in the British Navy.

Beriberi In 1882, Takaki found that he could stamp out beriberi in the Japanese Navy by certain changes in the diet He considered, however, that it was an increase in the protein intake which was responsible for the cure [12]

Theoretical Considerations Thus we may say that although dietary cures had been discovered for the control of these two diseases—beriberi and scurvy—yet the true nature of the dietary error responsible for them remained unknown It may nevertheless be recalled parenthetically that a far-sighted physician named Budd[13] had predicted in 1840 that scurvy is "due to the lack of an essential element which it is hardly too sanguine to state will be discovered by organic chemistry or the experiments of physiologists in a not too distant future "

Rickets Toward the end of the nineteenth century the view began to be expressed by pediatricians that "rickets is produced as certainly by a rachitic diet as scurvy by a scorbutic diet "[14] The use of cod-liver oil as a cure for rickets had been gaining ground steadily since the end of the eighteenth century

V. Deficiency Diseases Produced Experimentally

Experimental Beriberi In 1890 Eijkman,[2] in the Dutch East Indies, made the important discovery of experimental beriberi in fowls From 1890

[6] J Cartier, 1535 Quoted by R H Major, The Doctor Explains, Chapman and Hall, London, 1932

[7] [illegible]

[8] [illegible] 1639

[9] [illegible]

[10] [illegible]

[11] [illegible] 72–1775

[12] [illegible]

[13] [illegible] 40, p 99

[14] [illegible] subsequent

Vitamin A Similarly fat-soluble A was renamed vitamin A The most noticeable effects of its deficiency were found to include xerophthalmia, failure of growth, and increased liability to infection, especially of the respiratory system

Vitamin C It was apparent that the antiscurvy factor had entirely different chemical properties from either vitamin B or vitamin A It was accordingly given the next letter in the alphabet and became vitamin C

XI. The Growth of the Vitamin Alphabet

Vitamin D A little later, experiments on dogs[30] established the accuracy of the view that rickets was caused by the absence of a fat-soluble vitamin—present, for example, in cod-liver oil—which had long been recognized as an effective antidote At first it was natural to assume that this vitamin was identical with vitamin A, which was already known to be present in the curative agent, cod-liver oil Later, however, differences in distribution and chemical properties began to become apparent,[31] and it was accordingly named vitamin D

Vitamin E A new factor, vitamin E, needed to ensure normal reproduction in the rat, was described by Evans and Bishop[32] in California in 1922 Independent evidence of this new antisterility vitamin was obtained almost simultaneously by Sure[33] and by Mattill,[34] also in America

XII. The Vitamin B Complex

Vitamins B_1 and B_2 In 1926 Goldberger and his colleagues[35] proved, as Funk had predicted, that pellagra was associated with the lack of a vitamin He showed that it had a distribution somewhat similar to that of the antiberiberi factor but was more stable to heat He called it the P-P (pellagra-preventing) factor

An official committee in England,[36] however, revised the nomenclature The antiberiberi vitamin was renamed vitamin B_1, and the heat-stable component—then assumed to be a single substance—was called B_2 Vitamin B_2 was defined as the heat-stable, water-soluble factor, present in yeast extracts, needed to prevent pellagra in man and the apparently similar diseases in dogs (canine black tongue) and in rats (pellagra-like dermatitis),

[30] E Mellanby, *J Physiol (London)* **52**, Proc xi, liii (1918)

[31] E V McCollum, N Simmonds J E Becker, and P G Shipley, *J Biol Chem* **53**, 293 (1922)

[32] H M Evans and K S Bishop, *Science*, **56**, 650 (1922)

[33] B Sure, *J Biol Chem* **58**, 681, 693 (1923–1924)

[34] H A Mattill and R E Conklin, *J Biol Chem* **44**, 137 (1920)

[35] J Goldberger, G A Wheeler, R D Lillie and, L M Rogers, *U S Pub Health Rept* **41**, 297 (1926)

[36] Accessory Food Factors Committee, Minutes of Meeting, November 14th, 1927.

Noeggerath,[25] and Jacob.[26] The eminent Dutch physiologist Pekelharing[27] published, in 1905, the statement—generally overlooked at the time—that the unknown substances must be effective in very minute amounts, for he had found that quite small supplements of natural foods added to the artificial ration were sufficient to afford protection.

An independent and more detailed study by Hopkins[1] was aimed at showing (a) that an insignificantly small addition of milk would suffice to render the purified diet adequate and (b) that the animals ceased to grow while still eating sufficient in quantity of the purified diet to support good growth

VIII. The Revolution in Dietary Theory

The year 1912 marked the beginning of the new developments in dietary studies and the modern intensive work on vitamins. Hopkins's[3] celebrated paper on the inadequacy of purified diets, and Funk's[4] review on dietary deficiency diseases—avitaminoses—published a few months earlier, for the first time attracted world-wide attention to the vitamin question Nevertheless, for a few years to come, the existence of vitamins was still doubted by some

In 1929 the importance of the pioneer experiments of Eijkman and of Hopkins was recognized by the award to them jointly of the Nobel Prize for Medicine

IX. Differentiation of Fat-Soluble and Water-Soluble Vitamins

Fat-Soluble A and Water-Soluble B. The first real indication of the multiplicity of vitamins came in 1915 when McCollum and Davis[28] in America showed that, with the rat as an experimental animal, at least two accessory factors were needed for growth

One was present in fatty and the other in non-fatty foods. These were named, respectively, 'fat-soluble A' and 'water-soluble B'

X. Separating and Naming the Vitamins

Vitamin B. Before long it became recognized that what was called water soluble B had the properties of the antineuritic (antiberiberi) vitamine. To avoid confusion the two systems of nomenclature were combined At the suggestion of J C Drummond[29] water-soluble B was renamed vitamin B the terminal -e of the word vitamine being omitted to avoid any unwarranted implication that it necessarily had the chemical nature of an amine

Vitamin A Similarly fat-soluble A was renamed vitamin A The most noticeable effects of its deficiency were found to include xerophthalmia, failure of growth, and increased liability to infection, especially of the respiratory system

Vitamin C. It was apparent that the antiscurvy factor had entirely different chemical properties from either vitamin B or vitamin A It was accordingly given the next letter in the alphabet and became vitamin C

XI. The Growth of the Vitamin Alphabet

Vitamin D A little later, experiments on dogs[30] established the accuracy of the view that rickets was caused by the absence of a fat-soluble vitamin—present, for example, in cod-liver oil—which had long been recognized as an effective antidote At first it was natural to assume that this vitamin was identical with vitamin A, which was already known to be present in the curative agent, cod-liver oil Later, however, differences in distribution and chemical properties began to become apparent,[31] and it was accordingly named vitamin D

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[34] H A Mattill and R E Conklin, *J Biol Chem* **44**, 137 (1920)

[35] J Goldberger, G A Wheeler, R D Lillie and, L M Rogers, *U S Pub Health Rept* **41**, 297 (1926)

[36] Accessory Food Factors Committee, Minutes of Meeting, November 14th, 1927

and needed also for promoting the growth of rats. Vitamin B_2 itself, as so defined, however, soon proved to be complex.

XIII. The Vitamin B_2 Complex

Riboflavin. The first component of the B_2 complex to be characterized was the substance riboflavin, previously known as a naturally occurring yellow pigment in milk, which in 1933 was shown to possess growth-promoting activity for rats.[37] Riboflavin is, therefore, sometimes still referred to simply as vitamin B_2.

It was later found that a deficiency of riboflavin may be the cause of cheilosis (lesions on the lips) in man.

Vitamin B_6 (Pyridoxin). Soon afterwards, in 1934, a second component of the vitamin B_2 complex, at first called vitamin B_6,[38] but later renamed adermin, and now generally known as pyridoxin, was identified, also by experiments on rats. Its absence from their diet caused severe skin lesions.

Nicotinamide. In 1935, Birch, Gyorgy, and Harris[39] showed that the *pellagra-preventing factor proper was a third component, distinct from* both riboflavin or vitamin B_6. In 1937, this P.-P. vitamin was identified[40] with nicotinic acid, or nicotinic amide (sometimes also known in America as niacin, or niacin amide, respectively).

XIV. The Discovery of Additional Vitamins

Year by year additional vitamins were coming to light, as listed below. At least three of these have proved of importance in human nutrition, viz., vitamin K, folic acid, and vitamin B_{12}. Several others are considered to be needed, at any rate, by man. Some of the remainder have so far been studied only experimentally in animals, and their significance for the human thus remains largely unknown. It will be recalled, too, that several of these vitamins are also needed as growth factors by microorganisms.

The list that follows is in approximate chronological order.

(1) "*Vitamin F*." Nutritionally essential unsaturated fatty acids (linoleic, linolenic, and arachidonic acids) needed by rats.[41, 42] A deficiency causes a scaly tail, irregularities in reproduction, and kidney lesions.

(2) *Pantothenic Acid.* Prevents a specific dermatitis in chicks, formerly called, somewhat misleadingly, chick pellagra,[43] and identical with a so-

[37] R. Kuhn, P. Gyorgy, and T. Wagner Jauregg, *Ber.* **66B**, 317, 576 (1933).
[38] P. Gyorgy, *Nature* **133**, 498 (1934).
[39] T. W. Birch, P. Gyorgy, and L. J. Harris, *Biochem. J.* **29**, 2830 (1935).
[40] C. A. Elvehjem, R. J. Madden, F. M. Strong, and D. W. Woolley, *J. Am. Chem. Soc.* **59**, 1767 (1937).
[41] G. O. Burr and M. M. Burr, *J. Biol. Chem.* **82**, 345 (1929).
[42] H. M. Evans, S. Lepkovsky, and E. A. Murphy, *J. Biol. Chem.* **106**, 431, 441, 445 (1934).
[43] L. C. Norris and A. T. Ringrose, *Science* **71**, 643 (1930).

called filtrate factor for rats Its deficiency in man, may, apparently, sometimes be the cause of glossitis, and perhaps of an endemic disease (burning-feet syndrome) seen in Southern India

(3) *Vitamin H or Biotin* First known as the 'H' or 'Haut' (= skin) factor for rats [44] Biotin, as it is now called, has been shown to be needed by human beings, although it has not yet found any regular use in clinical medicine In chicks, likewise, a deficiency of biotin has been found to cause dermatitis

(4) *Choline* Concerned in fat metabolism in rats,[45] [46] and a biological transmethylating agent A deficiency in rats causes abnormalities in liver, kidneys, and elsewhere

(5) *Vitamin K* First recognized by Dam[47] in 1935, as concerned in preventing hemorrhages in chicks, and later shown to have clinical applications in the cure of certain hemorrhagic disorders due to a condition of hypoprothrombinemia In other words, vitamin K is needed for the elaboration in the liver of the prothrombin, concerned in the clotting of the blood

(6) *"Vitamin P"* (flavone glycosides) Supposed permeability (= capillary fragility) factor for guinea pigs,[48] human relations in dispute

(7) *Folic Acid (Pteroylglutamic Acid)* A factor, whose recognition dates from about 1941, of interest in connection with certain types of macrocytic anemia, e g , it has been successfully used in the treatment of tropical sprue in man

(8) *Inositol* Needed for the growth of various microorganisms[49] and said to prevent loss of hair in rats and mice [50] [53]

(9) *p-Aminobenzoic Acid* Growth factor for certain microorganisms, antagonized by sulfonamide drugs [54] Said to be needed to maintain a normal pigmentation of the hair—i e , to prevent it greying—in some species [55]

(10) *Strepogenin* Growth factor for certain hemolytic streptococci, considered also to promote growth in mice and guinea pigs[56] and perhaps in rats

[44] P György, *Z arztl Fortbildung* **28**, 377, 417 (1931)

[45] C H Best J M Hershey, and M E Huntsman, *Am J Physiol* **101**, 7 (1932), *J Physiol* **75**, 56 (1932)

[46] C H Best, M E Huntsman, E W McHenry, and J H Ridout, *J Physiol*, **84**, 38P (1935)

[47] H Dam, *Nature* **135**, 652 (1935), *Biochem J* **29**, 1273 (1935)

[48] S Rusznyak and A Szent György, *Nature* **138**, 27 (1936)

[49] E V Eastcott, *J Phys Chem* **32**, 1094 (1928)

[50] D W Woolley, *J Biol Chem* **136**, 113 (1940)

[51] D W Woolley, *Science* **92**, 384 (1940)

[52] P L Pavcek and H M Baum, *Science* **93**, 502 (1941)

[53] E Nielson and A Black, *Proc Soc Exptl Biol Med* **55**, 14 (1944)

[54] D D Woods, *Brit J Exptl Path* **21**, 74 (1940)

[55] S Ansbacher, *Science* **93**, 164 (1941)

[56] D W Woolley, *J Exptl Med* **73**, 487 (1941)

(11) *Animal Protein Factors* Associated with proteins of animal origin, and stimulating the growth of animals restricted to certain experimental diets [57, 58]

(12) *Vitamin B_{12}* One of the animal protein factors, first investigated in the United States as a growth factor for a particular microorganism,[59] and almost immediately afterwards identified as identical with the anti-pernicious-anemia factor, first recognized by Minot and Murphy,[60] and isolated independently and virtually simultaneously in 1948 by E Lester Smith[61, 62] in England A point of special interest is that its molecule contains the mineral element cobalt

XV. Chemistry of the Vitamins

Chemical Constitution and Synthesis During the decade 1928 to 1938, as a result of intensive effort by hundreds of investigators, the better-known vitamins (including A, B_1, C, D, and E) were isolated in a state of purity, one by one, and their chemical constitutions were then worked out Within a few years most of these (vitamins A, B_1, C, and E) had been synthesized in the laboratory, and the artificial products proved to be identical in properties and physiological effect with the natural ones.

Later, other vitamins were in turn to be similarly characterized and synthesized—or, in some cases (e g, nicotinic acid, choline, inositol, *p*-aminobenzoic acid), they were proved to be identical with substances which had previously been known to chemists but had not yet been recognized to possess vitamin activity

Examples of Chemical Names and Chemical Relations Vitamin A ($C_{20}H_{29}OH$) was shown to be chemically related to the naturally occurring hydrocarbon β-carotene ($C_{40}H_{56}$) which shares its biological activity

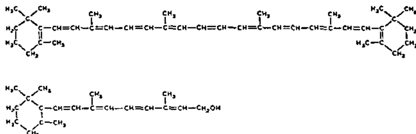
Vitamin B_1 ($C_{12}H_{17}N_4OSCl \cdot HCl \cdot$) has been given the name of aneurin (or thiamine in the United States)

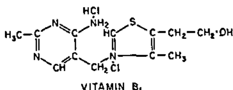
Vitamin C, ascorbic acid, has the empirical formula $C_6H_8O_6$

There are several forms of vitamin D, the most important being calciferol or vitamin D_2 ($C_{28}H_{43}OH$) and vitamin D_3 ($C_{27}H_{43}OH$)

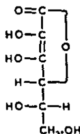
Vitamin E, α-tocopherol, has the formula $C_{29}H_{50}O_2$, there are also β-, γ-, and δ-tocopherols, with somewhat lower biological potency (for rats)

[57] C A Cary, A M Hartman, L P Dryden, and G D Likely, *Federation Proc* **5**, 128 (1946)
[58] A M Hartman, *Federation Proc* **5**, 137 (1946)
[59] M S Shorb, *J Biol Chem* **169**, 455 (1947)
[60] G R Minot and W P Murphy, *J Am Med Assoc* **87**, 470 (1926)
[61] E Lester Smith, *Nature* **161**, 638 (1948)
[62] E Lester Smith, *Nature* **162**, 144 (1948).

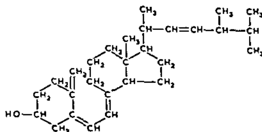




β-CAROTENE (above), showing its relation to VITAMIN A (below)

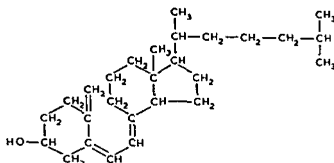


VITAMIN B_1

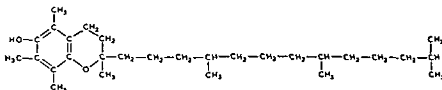


VITAMIN C (L-ASCORBIC ACID)

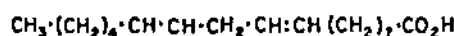
CALCIFEROL (VITAMIN D_2)



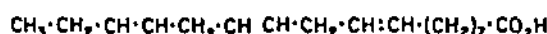
VITAMIN D_3



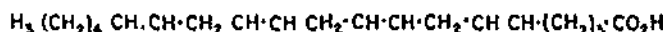
VITAMIN E (α-tocopherol)



Linoleic acid (Octadeca-9,12-dienoic acid)

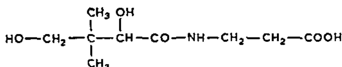


Linolenic acid (Octadeca-9,12,15-trienoic acid)

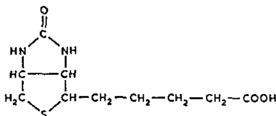


Arachidonic acid (Eicosa-5,8,11,14-tetraenoic acid)

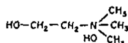
THE GROUP OF "F" VITAMINS
(i.e. Nutritionally essential, unsaturated fatty acids)



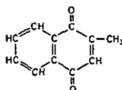
PANTOTHENIC ACID
(α,γ-Dihydroxy-β,β-dimethyl-butyryl-β-alanide)



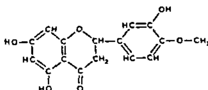
BIOTIN (VITAMIN H)



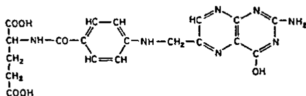
CHOLINE
(Trimethylhydroxyethyl-ammonium hydroxide)



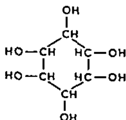
VITAMIN K_3 — 'MENAPHTHONE' ('MENADIONE')
(2-Methyl-1, 4-naphthoquinone)



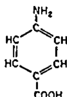
VITAMIN P
(Hesperitin, in the form of its glycoside,
Hesperidin, is regarded as one form of VITAMIN P)



PTEROYL-GLUTAMIC ACID



INOSITOL
(Cyclohexanehexol, benzene hexahydroxide)



p-AMINOBENZOIC ACID

XVI. Biochemical Research on Vitamins

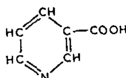
Side by side with these investigations on the organic chemistry of the vitamins, methods were being elaborated by which vitamins could be accurately assayed in biological materials or in foodstuffs, and the losses incurred on cooking or processing ascertained; their chemical and physical properties were being examined in close detail, tests were devised to assess the status of a human subject in some particular vitamin; the daily requirements were being estimated, some new and unexpected clinical uses were found, and the mode of action of the vitamins in the body was being gradually elucidated

XVII. Vitamins and Coenzymes

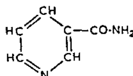
The most notable advance in our understanding of the chemical rôle of the vitamins has been the discovery that various vitamins of the B group owe their biological activity to the fact that they can function as coenzymes in the living cell. The pioneer observation of this kind was the finding of Lohmann and Schuster[63] in 1937 that the pyrophosphate ester of vitamin B_1 is the coenzyme for decarboyxlation of pyruvic acid, a substance which is an important metabolite in the breakdown of carbohydrates

With regard to other B vitamins, later knowledge has shown that nicotinamide functions as a coenzyme in the pyridine dinucleotide systems; riboflavin in the flavin dinucleotides; pyridoxin in codecarboxylase, and pantothenic acid in an acetylation coenzyme.

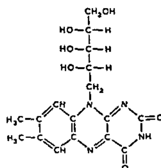
[63] K Lohmann and P Schuster, *Biochem Z* **294**, 188 (1937).



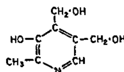
NICOTINIC ACID



NICOTINAMIDE



RIBOFLAVIN
(6,7-Dimethyl-9-D-ribityl-*iso*alloxazine)



PYRIDOXINE (VITAMIN B_6)
(2-Methyl-3-hydroxy-4,5-di(hydroxymethyl) pyridine

XVIII. Practical Implications of Modern Knowledge of Vitamins

It may be useful to conclude with a general statement, in elementary terms, of the practical upshot of this growth of knowledge about vitamins.

Clinical Relations Of the twenty vitamins now recognized, at least eight are known to be needed by man, or to be of undoubted clinical significance·

(1) *Vitamin A is used in the treatment or prevention of xerophthalmia* or certain other xerotic manifestations (xerosis and metaplasia in mucous membrane tissues)

(2) Vitamin B_1 is effective in the treatment of beriberi, a disease preva-

lent in rice-eating regions of the globe, or in the treatment of cases of conditioned polyneuritis, occasionally seen in hospital practice, and caused by a faulty absorption of the vitamin (see below, p 34)

(3) Vitamin C cures or prevents scurvy, a disease marked by hemorrhagic manifestations (including spongy, bleeding gums), and associated with the use of a diet containing inadequate amounts of fresh fruits, or vegetables, or other fresh foods

(4) Vitamin D is the vitamin which cures or prevents rickets, previously an extremely common disease of infancy, manifested in the softening and deformity of the bony structures of the body.

(5) Vitamin K is used in the treatment of a particular type of hemorrhagic disorder, associated with a low prothrombin value of the blood—notably in the newborn and in conditioned deficiencies, e g., in obstructive jaundice.

(6) Vitamin B_{12} cures pernicious anemia and some other varieties of macrocytic, megaloblastic anemias.

(7) Folic acid is effective in certain other types of macrocytic, megaloblastic anemias—e g , tropical sprue

(8) Nicotinamide prevents or cures pellagra, a disease endemic particularly in maize-eating areas, and characterized by a special kind of dermatitis and other signs

(9) Riboflavin may have occasional use in the treatment of certain types of dermatitis and oral lesions

Practical Significance of Other Vitamins There are at least seven other vitamins the exact significance of which in practical human dietetics still remains in some doubt These are pyridoxin, pantothenic acid, biotin, choline, vitamin E, vitamin F, and vitamin P

Another three have been demonstrated so far only by experiments on animals or microorganisms These are inositol, *p*-aminobenzoic acid, and strepogenin

Various Forms and Names Most of the vitamins exist in more than one form or modification For example, vitamin A_1 and β-carotene are two of the more important forms of vitamin A, and vitamins D_2 and D_3 of vitamin D Most of the vitamins have also been given more exact chemical names for each of their several forms, and (unfortunately)sometimes several synonyms Thus crystalline vitamin D_2 is called calciferol, and vitamin B_1 was (until recently) known as aneurin in Europe or thiamine in America. Thiamine is now official Similarly, nicotinamide was until recently known

ferent vitamins which they contain

For example, halibut-liver oil is extremely rich (and cod-liver oil some-

what less rich) in vitamins A and D, but it contains no trace of vitamins B, C, or E

Certain fruits—for example, oranges, lemons, and black currants—are valuable sources of vitamin C, but they contain little or no vitamins A or D Apples and plums, on the other hand, are less good for vitamin C Again, different types of apples vary in the amount of this vitamin which they contain

Wheatmeal bread is a good instance of a food well supplied with vitamin B_1, but there is little in white flour, white bread, or biscuits, and these are all devoid of vitamins C and D

Milk is an example of a food which contains all the vitamins so far mentioned (and others in addition), although it is richer in some of them than in others Thus it contains relatively little vitamin E

Vitamin Values It is scarcely ever possible to give an absolute value for the vitamin content of a food, but only an average value or range For example, Seville oranges as purchased in Britain contain on the average around 35 mg of ascorbic acid per 100 g, but individual specimens may contain anything from 20 to 45 mg

Effect of Processing, etc In addition, a variable degree of destruction of the different vitamins may occur as a result of ageing, storage, handling, processing, and cooking of the foods This will depend on the conditions employed and on the vitamin in question For instance, an average value for vitamin C in new potatoes is 45 mg per 100 g for the variety King Edward, and 25 mg for Majestic Old potatoes may contain only one-third to one-half as much as new potatoes Cooking by boiling may cause an average loss of about 60%

Vitamin Requirements A certain minimum quantity of each vitamin is needed in our diet if we are to be protected from the corresponding vitamin-deficiency disease For example, a diet containing no fresh fruit or vegetables, and consisting only of such foods as dried fish, dried meat, bread and butter, cakes and pastries, tea, cocoa, sugar, and condensed milk, is devoid of vitamin C An adult restricted to it would develop scurvy in about 6 to 9 months If, however, any food containing enough vitamin C to supply about 10 to 20 mg per day were to be included in this diet (or the same amount of the pure crystalline substance were to be administered), scurvy would be prevented or cured, and similarly for the other vitamins

Daily Minimum Requirements The daily minimum dose varies greatly from one vitamin to another It is usual to add a small margin for safety and for human variability, and, with this proviso, the reputed daily requirements range from 30 mg per day for vitamin C, down to 1 mg for vitamin B_1, or 0 01 mg for vitamin D

Factors Affecting the Requirement The foregoing figures give the re-

quirements in approximate round numbers, and it would be a mistake to apply them over-rigidly Indeed the amount of a given vitamin needed may vary with other factors. Thus the requirement for vitamin B_1 depends on the carbohydrate intake, and for vitamin D on the calcium and phosphate, and other things. The value cited above for vitamin D applies to a child, adults appear to need much less

Vitamins in Practical Dietetics The type of diet consumed in normal circumstances by most middle-class adults in Britain, as well as in most parts of Europe and America for example, can be relied on to supply a sufficiency of all the vitamins, provided that it is reasonably varied ('a good mixed diet') This is not to say that vitamins are, after all, of little practical significance. Their discovery has been of immense consequence and has made possible the conquest of diseases such as rickets, formerly very common indeed in Britain, and of scurvy, beriberi, and pellagra, scourges in other regions of the globe Indeed a good deal of mild rickets still occurs in Britain, America, and elsewhere, because of a neglect to supplement children's diets with adequate vitamin D.

Conditioned Deficiency As already hinted, a vitamin-deficiency disease may sometimes result, not because of a deficiency in the diet, but because the patient is unable to ingest or absorb the vitamin This is called a conditioned deficiency

Examples are the polyneuritis (= beriberi), or the pellagra-like symptoms, that are from time to time found associated with gastrointestinal obstruction or with alcoholism, and which are caused by the inability of the patient to ingest (or to utilize or assimilate) the vitamin B_1 or nicotinamide, respectively

Similarly, a deficiency of vitamin K, manifest as a hemorrhagic disease, is generally conditioned by failure of absorption.

Chemical Classification of the Vitamins In the early days of vitamin research, a somewhat arbitrary but useful distinction was made between the fat-soluble and the water-soluble vitamins. The distinction is based on the fact that the former are found to occur in fatty foods only, or in the fatty portions of food, and the latter in the non-fatty foods or fractions. Similarly, the fat-soluble vitamins dissolve in the so-called fat solvents (e g , ether, light petroleum), whereas the water-soluble vitamins are generally soluble in various aqueous media, including dilute acids and mixtures of alcohol and water.

Five of the vitamins are fat-soluble, viz , vitamins A, D, E, F, and K. The remainder are water-soluble

Chemical Properties The vitamins differ widely from one to another in their chemical nature and possess few, if any, chemical features in common. In one respect, however, namely, in their biochemical mode of action,

several of the water-soluble vitamins are recognized to function as coenzymes, as has been mentioned above This consideration suggests a possible alternative definition for a vitamin (cf above, p 18), namely, that "a vitamin is an exogenous catalyst, that is, one which the animal organism needs but is unable to synthesize and must, therefore, receive in its food "

Differences between Species Species vary considerably in their needs for the different vitamins. For example, human beings need daily approximately 0 02 mg of vitamin C for every 100 g of body weight, but the guinea pig needs about forty times as much (i e , 0 8 mg per 100 g) Monkeys also require vitamin C, but other animals, including rats, mice, cats, dogs, horses, cattle, and sheep, do not need vitamin C in their diets, the reason being that they are able to synthesize it in their bodies

Microsynthesis of Vitamins Certain species, particularly ruminants (including such farm animals as cattle and sheep), thrive in the absence of B vitamins from their diet, whereas other species, such as humans, rats, and dogs, develop fatal deficiency diseases—beriberi, pellagra, etc. The reason appears to be that the first-mentioned group obtain these vitamins from the alimentary canal where they are elaborated by microorganisms, particulary by those associated with the massive processes of fermentation which occur in the rumen

Vitamins in the Plant Kingdom Several of the vitamins are known to play an important role in plant physiology Vitamin-like substances needed specially by higher plants are named auxins Again, vitamin-like substances are needed by bacteria and by other microbes (growth-promoting factors for microorganisms), and these may or may not be vitamins for mammals

Determination of Vitamins Three types of method have been used for determining the amount of a particular vitamin present in a foodstuff, or animal vegetable tissue, namely, (a) chemical or physicochemical, (b) biological, and (c) microbiological

An example of a physicochemical method is the determination of vitamin A by its ultraviolet absorption spectrum, and of carotene by its natural yellow color

An example of a simple chemical method is the determination of vitamin A by the production of a blue color with antimony trichloride, or of vitamin C by the decolorization of an indophenol derivative

Microbiologically, nicotinamide and riboflavin have been assayed by measuring the growth, or the lactic acid production, in the organisms *Lactobacillus arabinosus* and *Lactobacillus casei*, respectively

In biological assays, rats have been the species most commonly used, except that guinea pigs have to be used for vitamin C. Pigeons, chicks, dogs, pigs, and other species have all been used for special purposes The

criteria used is the prevention, or the cure, of the ill effects caused by the absence of the particular vitamin—sometimes a failure in growth or loss in weight, sometimes a more specific, pathological abnormality

RECAPITULATION AND SUPPLEMENT*

CHRONOLOGICAL CHART OF SOME OUTSTANDING POINTS IN THE HISTORY OF VITAMINS

I *Empirical Cure of Deficiency Diseases in Man*

1601 Scurvy cured by fresh fruit or vegetables (Lancaster[7])

1882 Beriberi cured by balanced diet, less rice (Takaki[12])

1782–1900*ca* Rickets cured by cod-liver oil (Darley, Percival,[64] Trousseau,[65] Guerin,[66] Bland Sutton, etc)

II *Production of Experimental Dietary Deficiency Diseases in Animals*

1890 Beriberi in hens (Eijkman[8])

1907 Scurvy in guinea pigs (Holst and Frölich[16])

1918 Rickets in dogs (Mellanby[30])

III *Theoretical Deductions*

1840 Antiscorbutic factor postulated (Budd[13])

1901 An antiberiberi factor demonstrated (Grijns,[14] Eijkman)

1906 Importance of minimal qualitative factors in diet emphasized, e g , in relation to scurvy and rickets (Hopkins[17])

1907 First work on characterization of a specific antiscorbutic factor (Holst and Frolich[16])

1912 The 'vitamine' hypothesis i e , antiberiberi, antiscurvy, antirickets, and antipellagra vitamines postulated (Funk[4])

IV *Independent and Contemporary Experiments on Composition of Natural Diets*

1881 Purified basal diets, i e , synthetic diets, proved to be inadequate (Lunin[1])

1905 Synthetic diets shown to be satisfactory if supplemented by small addition of milk (Pekelharing[27])

1909 Natural diets made inadequate by extraction with solvents (Stepp[20])

1912 Further confirmatory observations on 'synthetic diets' and their supplementation (Hopkins[3])

V *Recognition of Existence of Specific Protective Factors (Now Called Vitamins) Preventing the Several Classical Dietary Deficiency Diseases*

1901 Recognition of an antiberiberi factor (Grijns,[15] Eijkman), later called vitamin B (and still later vitamin B_1)

1907 Recognition of an antiscurvy factor (Holst and Frölich[16]), later called vitamin C

1915 Fat-soluble A (later called vitamin A) distinguished from water soluble B (later called vitamin B) (McCollum and Davis[18])

1918 Recognition of an antirickets factor (Mellanby[30])

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* The arrangement of this chart under a series of eight main headings has necessarily entailed an appreciable amount of chronological overlapping and of some repetition in the different sections

[64] T Percival, Essays Medical, Philosophical and Experimental, London, 1789, Vol II, 4th ed , p 354

[65] A Trousseau, Clinique Médicale de l'Hôtel Dieu de Paris, 2nd ed , Paris, 1865

[66] J Guerin, *Gaz méd* 16, 332 (1838)

1922 Differentiation of the antirickets factor, later called vitamin D, from vitamin A (McCollum et al [31])
1926 Recognition of an antipellagra factor (Goldberger et al [35]), and differentiation from the antiberiberi vitamin
1927 Designation of vitamin B_1 for the antiberiberi vitamin and B_2 for the heat stable fraction (later, however, shown to include other vitamins as well as the pellagra-preventing factor) (Accessory Food Factors Committee[36])

VI *Recognition of Existence of Additional Vitamins*

1922 Vitamin E (Evans and K S Bishop[32])
1933 Riboflavin (Kuhn, Gyorgy, and Wagner-Jauregg[37])
1934 Vitamin B_6, pyridoxin (Gyorgy[38])
1929–34 "Vitamin F" (Burr and Burr [41] Evans et al [42])
1931–39 Pantothenic acid (Ringrose et al [47] R J Williams[48])
1931 Biotin (Gyorgy[41])
1932–35 Choline (Best et al [43] [44])
1935 Vitamin K (Dam[47])
1936 "Vitamin P" (Rusznyak and Szent Gyorgyi[48])
1938–41 Folic acid (Mitchell, Snell, and Williams,[49] and others)
1928–40 Inositol (Eastcott,[49] Woolley[50] [51])
1940 p-Aminobenzoic acid (Woods[54])
1941 Streptogenin (Woolley[55])
1946 Animal protein factors (Cary et al,[57] Hartman[58])
1947 Vitamin B_{12} (Shorb,[59] E Lester Smith[61] [62])

VII *Isolation, Identification, and Synthesis of the Vitamins*

1926 Vitamin B_1 isolated (Jansen and Donath[70])
1931 Vitamin D (D_2) isolated (Askew et al,[71] Windaus[72] [73])
1932 Vitamin C identified (Svirbely and Szent-Györgyi[74] [75])
1933 Vitamin C synthesized (Ault et al,[76] Reichstein et al [77])
1936 Vitamin B_1 synthesized (Williams and Cline,[78] Andersag and Westphal,[79] Todd and Bergel[80])
1936 Vitamin E isolated (Evans et al [81])

[67] A T Ringrose, L C Norris, and G F Heuser, *Poultry Sci* **10**, 166 (1931)
[68] R J Williams, *Science* **89**, 486 (1939)
[69] H K Mitchell, E E Snell, and R J Williams, *J Am Chem Soc* **63**, 2284 (1941)
[70] B C P Jansen and W F Donath, *Chem Weekblad* **23**, 201 (1926)
[71] F A Askew, H M Bruce, R K Callow, J St L Philpot, and T A Webster, *Nature* **128**, 758 (1931)
[72] A Windaus, *Nature* **128**, 39 (1931)
[73] A Windaus, *Proc Roy Soc (London)* **B108**, 568 (1931)
[74] J L Svirbely and A Szent-Györgyi, *Nature* **129**, 576 (1932)
[75] J L Svirbely and A Szent Györgyi, *Biochem J* **26**, 865 (1932)
[76] R G Ault, D K Baird, H C Carrington, W N Haworth, R W Herbert, E L Hirst, E G V Percival, F Smith, and M Stacey, *J Chem Soc* **1933**, 1419
[77] T Reichstein, A Grüssner, and R Oppenauer, *Helv Chim Acta*, **16**, 561, 1019 (1933), *Nature* **132**, 280 (1933)
[78] R R Williams and J K Cline, *J Am Chem Soc* **58**, 1504 (1936)
[79] H Andersag and K Westphal, *Ber* **70B**, 2035 (1937)
[80] A R Todd and F Bergel, *J Chem Soc* **1937**, 364
[81] H M Evans, O H Emerson, and G A Emerson, *J Biol Chem* **113**, 313 (1936)

	1937	Pellagra-preventing factor identified as nicotinamide (Elvehjem *et al*[40])
	1936	and onward Isolation, identification and synthesis of other vitamins
VIII	*Recognition of Vitamins as Components of Coenzyme Systems*	
	1932–35	Riboflavin and isoalloxazine coenzymes (Warburg and Christian,[82] Ellinger, Kuhn *et al*,[37] György,[38] Karrer and his collaborators)
	1935–37	[illegible]
	1937	[illegible]
	1946	Vitamin B_6 and codecarboxylase (Gale[87])
	1947	Pantothenic acid and acetylation coenzyme (Lipmann *et al*[88])

[82] O Warburg and W Christian, *Naturwissenschaften*, **20**, 688, 980 (1932); *Biochem Z* **254**, 438 (1932), **257**, 492 (1933), **266**, 377 (1933)

[83] H von Euler, H Albers and F Schlenk, *Hoppe-Seyler's Z. physiol Chemie* **237**, 1 (1935)

[84] O Warburg and W Christian, *Biochem Z* **275**, 464 (1935), see also O. Warburg, W Christian, and A. Griese, *Biochem Z* **279**, 143 (1935).

[85] B C J G Knight, *Biochem J* **31**, 731 (1937).

[86] J H Mueller, *J Bact* **34**, 429 (1937), see also J H Mueller, *J. Biol Chem* **120**, 219 (1937)

[87] E F Gale, *Proc Nutrition Soc* (*Engl. and Scot*) **4**, 130 (1946)

[88] F Lipmann, N O Kaplan, G. D Novelli, L C. Tuttle, and B M Guirard, *J. Biol Chem* **167**, 869 (1947)

CHAPTER 3

Water and Electrolyte Metabolism

R L ZWEMER

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I. Introduction

Water can be taken for granted as fundamental in nutrition and has been so recognized from the earliest records of man Almost as ancient is the record of need for salt (sodium chloride) in nutrition of animals and man Within the past half-century, potassium has also come to be regarded as playing an important part in the function of the living cell

Citation of the many research contributions to the subject of water and electrolytes would be overwhelming in contemplation and impossible within the limits set for this chapter This will be a concise presentation of current concepts derived from original research, reviews, books, and symposia. References which are given will permit the reader to find sources of information where the details of a particular subject may be examined more carefully

II. Water in the Living Body

1 General Considerations

Water is the largest single constituent of all living matter, plant or animal In living cells it amounts to about two-thirds, in supporting structures such as bone it may be only one-half, and in some body fluids more than nine-tenths of the material present

The true water content is difficult to determine exactly. This is due in part to the many conditions in which water is present in living things and in part to the fact that many of the compounds, dissolved in water or enclosing water, will yield or gain water during the course of an analysis. In spite of these difficulties, investigators have standardized their analytical procedures so that water content, as determined by different means, are quite comparable This is important since analyses of other constituents of living material are frequently closely related to the water present or to the water-free residue

One-celled animals and plants have the water of their protoplasm as their own and exert no great influence over the water outside the cell, except for excretions and small drops that they may enclose for a time in a vacuole With an increase in the numbers of cells in an organism there may be present a cavity or series of cavities which involve temporary enclosure of a small portion of the surrounding ocean, pond, or puddle In more complex forms of life the fluid outside the living cell, yet in contact with it, becomes an intimate part of the whole organism, and elaborate mechanisms exist to maintain its volume and composition

The living organism, both plant and animal, needs water, is largely made up of water, and the other components or products of living protoplasm—proteins, carbohydrates, fats, and minerals—are dissolved or suspended in water, hold or release water, or are otherwise closely identified with water in its various states Water can be obtained by living cells from contact in a fluid environment, from the air as water vapor, from water as a constituent of foods and drink, or as a product of metabolism of some other component of the body

Water is present in the body in free or continuous phase, as in plasma, lymph, or connective tissue fluid, or in a discontinuous phase in which the amount of water enclosed by cells and cell colloids becomes greatly dispersed This greater dispersion is associated with an increased firmness of the gel state of the colloid

2 Extracellular Fluid

Extracellular fluid—that which is found outside living cells—is generally considered to be a free-flowing liquid and, as far as the water component is concerned, freely exchangeable with other extracellular fluids. A possible

exception that must be kept in mind is the water in bone and cartilage that is not a part of the living cell protoplasm but rather is a constituent of the extracellular interstitial matrix

Within the body there is, in more complicated forms of animal life, a separation of extracellular fluid (*a*) Distribution of a circulatory fluid by heart action is in a closed system lined with a thin endothelium, backed up in larger vessels by various thicknesses of connective tissue and smooth muscle The nature of the membrane lining this capillary portion of this system permits the free passage of salts, sugars, and other small molecules but usually retains protein and other large molecules (*b*) Closely related is a one-way closed lymphatic system which receives fluid material from the tissue spaces and discharges it into the circulatory system at one or more points (*c*) Wherever living cells are not in direct contact with each other, within an organ or between organs and organ systems, there will be a loose mesh of supporting tissue with its spaces filled with fluid This tissue provides the means by which the living cell receives all those nutrients it needs for its existence, and into this fluid is discharged the waste products no longer needed by the cell It also serves as a reserve area in which water is stored or from which water may be withdrawn by changes in the relationship between osmotic and hydrostatic pressure

The relation of this free-flowing water to the rest of the water in the body may be illustrated by data for man The results obtained by the use of different methods of analysis and by subsequent interpretation vary somewhat so that only broad generally accepted values are given These values are compatible with each other and illustrate this relationship

Total water equals about 60% of body weight—4% in plasma, 14% extracellular, and 42% intracellular A 70-kg man will have about 2 8 l of blood plasma, 9 8 l of other extracellular fluid, and about 29 4 l of cellular water The 2 8 l of blood plasma will have about 2 1 l of red blood cells, giving a blood volume of 4 9 l or about 7% body weight Lymph, in a one-way closed system leading from tissue spaces to the venous system, has a variable volume dependent on many factors The interstitial fluid is widely distributed throughout the body, its distribution coinciding very closely with the loose web of connective tissue found between cell groups of organs, between organs and supporting structures, and other potential spaces (*d*) Closed spaces at joints contain synovial fluid and, in the brain case and spinal column, the central nervous system is surrounded by a cerebrospinal fluid of a different composition from plasma. (*e*) Special fluids in the form of secretions such as tears, saliva, and gastrointestinal fluids and their reabsorption represent a turnover from one form to another rather than an additional amount of fluid in the body. Excretions which represent a loss of water from the body include urine, sweat, milk, and the water component of feces

3 Intracellular Fluid

Intracellular fluid—that which is part of the protoplasm of the living cell—is difficult to study and almost impossible to obtain in pure form. The most easily studied cells are the red blood cells, which by centrifuge can be packed to exclude most of the extracellular fluid; however, they are specialized and are non-nucleated in those species on which most research has been done. Other living cells have a variable amount of connective tissue between and around them, and this holds extracellular fluid in its fine meshes In this case an analysis would give a mixed result from which the true cell water would need to be calculated Determination of the water content of the living cell is further complicated by the presence of inclusions within the cell The importance of fat (lipids) as a factor in complicating the determination of gases in body fluids has been recognized The fat cell in adipose tissue has only a thin membrane of living protoplasm enclosing the large fat globule or globules, and analyses show the water content of adipose tissue to be very low, about 30%.

4 Tissue Differences

The wide variation in proportion of living cells to extracellular material is reflected in the total water and electrolyte composition of tissues

a Those composed largely of living cells, such as the liver and the kidney, will have a large component of intracellular water with its typical composition of solutes The amount of extracellular tissue and water will be very small.

b Some tissues, such as bone, cartilage, and tendon, will have relatively *few cells and thus very little intracellular fluid Analyses of the material* laid down outside the living cells show that the mineral composition in relation to the water component is characteristically extracellular.

c In less compact tissues, such as thymus and uterus, there are portions in which the living cells occur in a loose mesh or network and the spaces between are filled with blood or lymph in vessels and interstitial fluid in the connective tissue spaces In these it would be very difficult to identify precisely the amounts of intra- and extracellular fluid

d. A fluid tissue, such as blood, is about two-fifths cells and three-fifths extracellular plasma under normal conditions

e Fat or adipose tissue with the low water content of 30% has a relatively small amount of extracellular fluid and of protoplasm with its intracellular fluid. The fat itself presents certain peculiarities which will be discussed later.

5 Determination of the Water Content

Determination of the water content of the body and its component parts has been approached in a number of ways.

The water may be removed by heat, by vacuum, or by both, and the initial weight minus the residue after drying to a constant value should be water. Other volatile substances and decomposition to produce water may occur. Another approach has been through the recovery of water distilled off from the tissue being analyzed.

a. The Dilution Principle

In the intact living individual reliance has been placed on analysis by the dilution principle.[1-4] Solutions of substances which can be identified later are injected into the fluid space to be measured. Time is allowed for uniform distribution, and the total volume is calculated from the degree of dilution. Large molecular substances that remain in the blood vessels for some time, such as Evans blue (T1824), have proved to be the best for determining plasma volume. Newer methods include tagged plasma protein with a radioactive component. Smaller molecules that leave the capillaries but are not actively retained by living cells, such as thiocyanate, thiosulfate, inulin, or radiosodium, have been satisfactory in determining extracellular fluid volume. The isotopes of hydrogen—deuterium and tritium—as components of water itself, have been useful in the determination of total water content of the body.[5,6]

It is important to note that many of the physiological and biochemical interpretations of data, based on water and its solutes, have failed to take into consideration the 10% to 30% of body weight of low water content fat deposits.

b. The Importance of Fat

The wide variation in water content of tissues reported by various investigations may be due in part to a failure to take fat into consideration. In obese individuals, both man and animals, the water content may be 50% and in lean individuals as high as 70%, and yet the water content may be a constant fraction of the fat-free body mass. Voit[7] very early showed that when fat was removed, fat and lean animals have closely related water con-

[1] N. M. Keith, L. G. Rowntree, and J. T. Geraghty, *Arch. Internal Med.* **16**, 547 (1915).

[2] I. S. Edelman, J. M. Olney, A. H. James, L. Brooks, and F. D. Moore, *Science* **115**, 447 (1952).

[3] M. F. Levitt, and M. Gaudino, *Am. J. Med.* **9**, 208 (1950).

[4] M. Gaudino, and M. F. Levitt, *J. Clin. Invest.* **28**, 1487 (1949).

[5] G. Hevesy, Radioactive Indicators, Their Application in Biochemistry, Animal Physiology and Pathology, Interscience Publishers, New York, 1948.

[6] N. Pace, L. Kline, H. K. Schachman, and M. Harfenist, *J. Biol. Chem.* **168**, 459 (1947).

[7] C. v. Voit, Physiologie des Stoffwechsels und der Ernährung. Handbuch der Physiologie, L. Hermann, Ed., 6(1), Vogel, Leipzig, 1881, pp. 345–415.

tents Studies of the lean body mass of man[8] and of body composition in guinea pigs[9] show a very close experimental relationship between specific gravity of the body and fat content. Considering fat as a dilutent of lean body mass, the water (73 2%) and combined nitrogen (3 52%) have been calculated to be relatively constant in a number of species of laboratory mammals[10] McCance[11] has stated that 71% water in the fat-free body of normal individuals is now an accepted constant

c. Other Considerations

Methods for analyzing the size of the various spaces or "compartments" of the body present difficulties because of interposition of living cells between the spaces being measured The cells may vary in their characteristics in different parts of the space and may break up the space into many smaller units Even blood in the closed tubes of the circulatory system has been found to vary in the proportion of red cells and in plasma composition in different parts of the system Cellular fluid is discontinuous by reason of the cell surfaces, two between each unit of cell water If the living cells are not in direct contact, then interstitial tissue and its extracellular fluid are also interposed between the units of cellular fluid. Cell water undergoes even greater dispersion, of course, because of the colloidal nature of protoplasm

These difficulties are enumerated to dispel the concept of neatly defined compartments which permit the addition or subtraction of water and solutes Nevertheless, a degree of accuracy has been attained which permits *comparable results on plasma volume, extracellular fluid volume, and total* body water

6 Osmotic Pressure

When a membrane is interposed between water containing one group of substances on one side and another group on the other, the mobility of the molecules involved works toward equalizing the distribution If the membrane permits the passage of particles of all sizes, the equalization is a function of concentration and time The membrane may allow passage of particles below a certain size If all the particles of a larger size are retained on one side, they exert a force (osmotic pressure) which continues until the water vapor pressure is equalized by drawing water toward the side of the membrane on which are located the majority of particles incapable of passing through the membrane Living membranes may complicate the physicochemical picture by reason of active metabolic transfer of particles that

[8] A R

[9] Edith

[10] N Pa

[11] R A McCance, B138, 115 (1951)

could or could not diffuse Osmotic pressure assumes major importance when we realize that the transfer of water in the body is conditioned to a large extent by the substances that are dissolved in it

One mole of an ideal solute, dissolved in 22 4 l of water, will exert a pressure of 1 atm at standard temperature When a solution becomes concentrated, as for example in cells with high protein and lipoid content, then the amount of water rather than the total volume of solvent and solutes must be used in calculating pressure In biological media osmolar concentration should be expressed as moles per kilogram of water

7 Structure of Water

The physical properties of water and of dilute aqueous solutions are intimately related to the functions of cells and of the living body In a careful review of work on the structure of water,[12] the conclusion was reached that water is not a randomly arranged mass of spherical molecules but rather is composed of "polar molecules coordinately arranged in some sort of lattice-like network, and bound by a number of intermolecular forces " Water in solid form as ice has regularity of structure, with a geometric arrangement of molecules In liquid form, water molecules may be coordinated in several forms, which may exist together and change from one to another, depending on temperature and the solutes present Unusual characteristics of water are its high heats of vaporization and fusion, great surface tension, internal pressure, and dielectric constant Its minimum specific heat at 37.5° and its great expansion on solidification have interesting physiologic relationships These physical properties are pertinent to discussions in Chapters 20–22, in which convection, circulation, heat capacity, and evaporation play a part

III. Electrolytes

1 Solutes in Body Fluids

In body water there are in solution a number of chemical elements These may be alone or in combinations as large as the protein molecules Only sodium, potassium, and chlorine will be discussed briefly in this chapter in relation to water in the body A number of excellent monographs give fuller treatment [13-16] Whenever the combinations exist as salts, it is proper to

[12] A S Crafts H B Currier, and C R Stocking Water in the P[illegible] Plants, Chronica Botanica Co , W[illegible]

[13] J P Peters, Body Water, Charles [illegible]

[14] A T Shohl, Mineral Metabolism [illegible]

[15] H L Marriott, Water and Salt Depletion, Charles C Thomas, Springfield, Ill , 1950

[16] H W Smith The Kidney Structure and Function in Health and Disease, Oxford University Press, New York, 1951

tents Studies of the lean body mass of man[8] and of body composition in guinea pigs[9] show a very close experimental relationship between specific gravity of the body and fat content Considering fat as a dilutent of lean body mass, the water (73.2%) and combined nitrogen (3.52%) have been calculated to be relatively constant in a number of species of laboratory mammals [10] McCance[11] has stated that 71% water in the fat-free body of normal individuals is now an accepted constant.

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[illegible] 97, 108 (1940 1941)

[11] R A McCance, and E M Widdowson, *Proc Royal Soc* (*London*) B138, 115 (1951)

2 Principle Cations and Anions in Body Fluids

As graphically shown by Gamble,[19, 20] the analyses of various body fluids show that in *cell fluid* the cations are mostly potassium, with some magnesium and smaller amounts of sodium and calcium, the anions are phosphate, sulfate, and protein with some bicarbonate *Extracellular fluids*, whether plasma, tissue fluid, or secretions, have sodium as the principle cation with small amounts of potassium, calcium, and magnesium, their anions are chloride and bicarbonate, with some protein in plasma The pH is usually regulated by the relative amounts of Cl^- and HCO_3^-, although in the acid gastric juice Na^+ is replaced to a considerable extent by H^+

IV. Water Metabolism

1 Internal Exchanges and Circulation

The movement and exchange of water and its solutes within the body is surprisingly great in the extracellular fluids that have been measured One can also infer, from the dilution curves of deuterium or tritium water used in analyses of total body water, and from studies of protein, fat, and carbohydrate metabolism, that an active exchange takes place between intra- and extracellular fluid

The hydrostatic pressure in the arterial part of capillaries, produced by the pumping action of the heart, forces fluid as an ultrafiltrate out into the tissue spaces. The large protein molecules which are retained in the capillaries exert osmotic pressure to draw similar fluid back into the capillaries at the venous portion Blood pressure and plasma protein content will alter respectively the amount which leaves and the amount which returns[21]

Intercellular fluid with components derived from the capillaries and from cell activity may enter the lymphatics and be returned to become part of the blood plasma again

Studies of lymph production, composition, and volume[22] show the way in which this interesting system returns tissue fluid to the blood stream in accordance with alterations in volume, brought about by fluid production through cell activity, or increased capillary filtration The latter is related to a higher blood pressure or lower protein osmotic pressure

In some tissues, such as adrenal cortex and liver, the capillary wall and

19 [illegible]

20 [illegible]

21 [illegible] Chicago, 1908

22 C K Drinker, and M E Field, Lymphatics, Lymph, and Tissue Fluid The Williams and Wilkins Co , Baltimore, 1933

assume that their reactivity will be that of the individual ions and not that of the molecules,[17] i e, $Na^+ + Cl^-$ rather than NaCl, and $K^+ + HCO_3^-$, not $KHCO_3$

Subsequent discussion of sodium and potassium will be based on their activity as individual cations. The anion chlorine is closely related to sodium in its activity. The bivalent cations calcium and magnesium are found for the most part in bone as complex salts, but a small amount is needed in extracellular fluid for the proper functioning of contractile tissue, especially the heart, nerve conduction, and other vital functions Further discussion of these and of phosphorus will be found in Chapter 21 The occurrence of sulfur in amino acids, proteins, glutathione, and thiamine and its oxidation to the sulfate ion are related to the problem of fixed-acid elimination in acid-base balance It is combined with sodium or potassium if these are available for excretion, otherwise ammonia formation is undertaken as a cation sparing activity

Iron as an essential part of hemoglobin and other cell chromatin substances is given special consideration elsewhere (Chapter 20). Another fourteen "trace" elements (Chapter 22) are present in minute amounts, and many of these are essential in animal nutrition Their absence or excess can bring about striking changes in a wide variety of glandular and tissue functions

Cells can accumulate ions against a gradient up to twenty-five times the external concentration The accumulation process is dependent upon metabolic energy and can be stopped by cyanide, O_2 deficiency, temperature variations, and limited food reserves It is limited in plants to growing cells Other solutes as well as electrolytes may be concentrated

Anomalous movements may be accounted for by water of hydration accompanying the solute One must not forget the further possibility that water, with its dipole character and hydrogen bonding, may enter into a structural relation of solute and solvent in a solution

Proteins are related to mineral metabolism and distribution by functioning as anions when on the alkaline side of their isoelectric point (Chapter 1, acid-base balance) They diminish water content because of their bulk and the space it occupies.

The ability of hydrophilic colloids to hold water, changes with salt concentration, it might therefore be possible to transfer water from one point and release at another by such solute changes. *Nitella* stimulated with sucrose will do this.[18]

[17] J Sendroy, Jr , *Ann Rev Biochem* **14**, 407 (1945)
[18] W J V Osterhout, *J Gen Physiol* **30**, 439–447 (1947)

and humidity. The structure of the skin surface, whether a few thin layers of cells or a thick horny layer, and feather and fur additions also influence water loss In man this can be conditioned by the surface area covered and the construction of the clothing worn The insensible loss, with little or no loss of salt, is in contrast with sweat which is hypotonic saline of from 0 1 to 0 5% Sweat appears when there is need to cool the body because of higher external temperature or increased heat production by work Since sweating is under nervous and hormonal influence, it may also appear under conditions of emotional stress

Excretion in sweat may be entirely absent at low temperatures A rare clinical condition has been reported in which there is an absence of sweat glands, and in these persons there is a rise in body temperature after exercise. At high temperatures or with heavy work, the volume of sweat may be from 2 to 4 l an hour, and the total for 24 hr may be more than 10 l

2 Output or Loss from the Body

As can be seen from the above discussion, the normal loss of water and salts from the body occurs through the insensible loss by way of skin and lung, the possible added surface loss by sweat, the small amount in the feces, and the regulated water and salt excretion in the urine The combined loss would be about 1 1 insensible loss, 0 1 l in feces, and 1 5 l in urine—a total of 2 6 l in 24 hr Under some conditions the loss by any one of these channels may exceed the total amount of extracellular fluid In spite of the ability of the body to use intracellular fluid by selective secretion of ions, it is obvious that the output must be balanced by intake, if serious consequences are to be avoided In pathologic conditions where there exists an inability to concentrate urine or selectively recover sodium or excrete potassium, the loss of fluid or the change in composition may be so great that life cannot continue

3 Requirements in Food and Drink

The normal requirement of water for an average adult would appear to be from 2 to 3 l. a day One milliter for each calorie of food taken has been presented as an ordinary standard[26] Most of this is present in prepared foods and the fluid supplements of normal diets

Fasting, when environmental conditions are favorable and a minimum of work is being done, still requires about 0 8 l of water per day This is about half the obligatory water requirement, but additional water will be provided by the oxidation of fat and protein and by the release of intracellular water by utilization of cell protein

[26] Food and Nutrition Board, Reprint and Circular Series No 129, National Research Council, Washington, D C, 1948

the cell wall are in intimate contact, and exchange of fluid, electrolytes, and other solutes can take place directly between cell and plasma without the intervention of intercellular fluids

Another type of discharge and absorption takes place in the digestive tract *As much as 8 l of fluid could be secreted in 24 hr by a combination* of tears, saliva (1 5 l), gastric juice (2 5 l.), bile (0 5 l), pancreatic juice (0 7 l), and other intestinal secretions (3 l)[15] Normally, most of this is absorbed back into the body together with the water and solutes of food and drink taken, and only about 0 1 l lost in the feces In constipation there is greater resorption and with diarrhea less, so that, especially in disease, the loss of water may be very serious

Within a single organ, the kidney, there is good experimental evidence to indicate a tremendous turnover of fluid[18] At the glomerular knot of thin-walled capillaries there is excreted a filtrate of dilute urine Additional substances are added and some constituents withdrawn during the passage down the renal tubule From experiments in dogs it has been estimated that glomerular filtrate was 100 to 300 ml per kilogram per hour If this holds for a 70-kg man, it would be 7 l of filtrate per hour, which is about two and one-half times the plasma volume, and over one hundred the normal urine volume of 1 to 1 5 l This filtrate-urine ratio would permit the very accurate regulation of the constancy of composition of extracellular fluid, which is so necessary for the normal functioning of cells. The urine excreted may contain less or more sodium, chlorine, and potassium than plasma, and the salt concentration may vary from 0 4 to 2% expressed as NaCl The ability to secrete a urine of a higher specific gravity than that of glomerular filtrate seems to be confined to those higher species that have a thin portion of the loop of Henle of the renal tubule

The cerebrospinal fluid system differs from plasma in having little or no protein, twice the magnesium concentration, and a slightly lower potassium content[23–25] The sodium content does not differ greatly if both are calculated on the basis of water present, but the chlorine is higher, to balance the lack of protein The cerebrospinal fluid is also slow in reflecting changes in blood plasma This "blood-brain barrier" offers good evidence for considering cerebrospinal fluid more of a secretion than a filtrate

Other small specialized amounts of extracellular fluid in the joints, eyeballs, *etc*, resemble plasma filtrates but differ sufficiently to also suggest selective secretion

The skin surface evaporation and loss by vapor in expired air amounts to about 1 l of water in 24 hr This is influenced by the external temperature

B Saunders, Phila-

J Y **7**, 297 (1938)

[25] R A McCance, and E Watchorn, *Brain* 57, 333 (1934)

The variations in potassium content of tissues should be correlated with the water of the living protoplasm of a particular tissue In those tissues having a larger proportion of non-cellular material the potassium content is low, but if the tissues could be individually analyzed it would still be high in the cells. Metabolic studies have shown that the destruction of cell protein results in the release of potassium, phosphorus, and nitrogen, all of which are excreted in the urine. In many cases the proportions are similar to those found in the tissues, from which it might be inferred that they are combined in the cell The need for potassium in growth has been noted both for young normal tissue and for rapidly growing, abnormal cancer cells This close relation between cell growth and breakdown and potassium loss and accumulation are strongly indicative of the dynamic role played by potassium in cell metabolism The excitability of protoplasm in the conduction of nerve impulses[33] and in the contraction of muscle[34] have been shown by extensive research to be markedly affected by the potassium level within the cell, by the amount of potassium in the extracellular fluid, and by the proportion of potassium to other cations, such as sodium, calcium, and magnesium

Carbohydrate metabolism, including the deposition of glycogen and the phosphate enzyme systems (Chapters 4 and 17), is known to be depressed and in some cases inhibited by a lack of potassium, and cells depleted of potassium require the presence of sugar for its restoration Enzymes and hormones, especially the adrenal cortex steroids, are now accepted as playing a critical role in potassium metabolism.[35]

Serious losses of potassium in the urine may occur through administration of excessive amounts of adrenocorticotrophic hormone of the anterior pituitary or cortisone, owing to their effect on the renal tubules Corticosteroid excretion and urine volume seem to be related, with a low corticosteroid excretion level being associated with a low urine volume and a large urine volume with normal or high levels of excretion [28,29]

Severe dehydration has also been found to result in potassium loss from cells without an equivalent loss of protein

The radioactive isotope of potassium (K^{42}) with a short half-life of 12.4 hr , has been used in the study of the ion permeability of red blood cells.[8,36] The experiments gave strong support to investigators who believed that the concentration gradients between high potassium and low sodium inside and high sodium with low potassium outside the cell are not the result of membrane impermeability

[33] K S Cole, *Cold Spring Harbor Symposia Quant Biol* 8, 110 (1940)
[34] H B Steinbach, *Ann N Y Acad Sci* 47, 849 (1947)
[35] R R Overman, *Physiol Revs* 31, 285 (1951)
[36] W O Fenn, *Physiol Revs* 20, 377 (1940).

When there are extreme external environmental conditions, combined with loss of intake, the reserve capacity of the body for adjustment may break down When no water is available there will be a deficit of about 2% of body weight per day. This is greatly increased as the external temperature goes up In desert conditions[27] it has been estimated that, although one could survive for about 10 days at 70°F., this would be shortened to 5 days at 100°F and perhaps 2 days at 120°F.

Illness *may exaggerate the loss of fluid if it involves severe vomiting,* uncontrolled diarrhea, or an increase of sweat because of high fever

Endocrine disturbances involving the adrenal glands, the pancreas, and the posterior pituitary, and toxemia of pregnancy can produce profound separate or combined effects on water and electrolyte metabolism.[29-31] In each of the many possible abnormal conditions it is important to determine whether the loss requires the replacement of water alone or as a solution of sodium chloride with perhaps added bicarbonate or potassium The sometimes lethal effects of giving the wrong solution have been emphasized by McCance and Young.[32] No single laboratory procedure should be depended upon for giving the degree of dehydration, but repeated determinations of hematocrit, plasma protein or specific gravity, and body weight may be valuable in determining the response to therapy.

It can be calculated that water requirements may be as high as 14 l a day, since this is the amount that can be lost either by sweat or by urine If the 8-l possible loss from the digestive tract is also considered, the total is three times that of all the extracellular fluid in plasma, tissue space, and elsewhere in the body The sensation of thirst is an excellent guide for water *intake, but the desire for fluids can be depressed by narcosis, drowsiness,* coma, or sodium chloride depletion

V. Principle Cations in Body Fluids

1 Potassium in Cell Water

Potassium is known by tissue analysis to be the most important cation of the living cell in both plant and animal tissues. It is recognized as playing an important part *in intracellular osmotic pressure In relation to cell* protein and the phosphate and sulfate anions it participates in the hydration of protoplasm and in the maintenance of acid-base balance in the cell

[27] E F Adolph, *Physiology of Man in the Desert*, Interscience Publishers, New York, 1947

[28] [illegible] (1951)

[29] [illegible] (1952)

[30] [illegible] (1952)

[31] [illegible]

[32] [illegible] (1944)

2 Potassium in Nutrition

Potassium, under normal circumstances, does not present a nutritional problem in animals. The plants and other animals which serve as food have potassium in those structures which are composed of living cells, so that the supply is ample for body needs. Any excess is taken care of in the normal selective process of elimination by the kidney.

Natural foods yield the minimum requirements, and the excretory mechanism of the body can easily take care of a fairly large excess. In a normal person a diet containing as much as 6 g. of potassium per day can be tolerated. There are conditions when a high potassium intake may be unwise and even dangerous, such as severe adrenal cortical insufficiency, damaged kidney function, or a lack of sodium intake coupled with a sodium loss. For these conditions it is possible to select a diet that is adequate in other respects, but which has a low daily potassium allowance.[43] Starvation, the therapeutic withholding of food, or the inability to take food may after several days result in a potassium deficiency. Patients subjected to operative procedures are more apt than others to develop potassium deficiency. They would have decreased intake by mouth and parenteral infusions which contain no potassium but which increase urine output. These solutions would also, because of their sodium content, displace potassium, and finally there may be gastrointestinal losses of potassium as well.

Percentages of Certain of the Mineral Elements in the Edible Portions of Foods*

	Potassium	Sodium		Potassium	Sodium
Almonds	0.759	0.003	Eggs	0.138	0.140
Apples	0.116	0.002	Ham, medium to lean	0.383	¶
Bananas	0.373	0.002	Lamb (mutton)	0.301	0.084
Beans, dried	1.201	‡	Milk (cow's)	0.143	0.051
Beans, snap or string	0.251	0.003	Oysters	0.201	0.471
Beef, lean	0.338	0.084	Potatoes	0.496	0.005
Bread, white	0.109	0.446	Prunes, dry	0.848	0.008
Bread, whole wheat	(0.45)†	§	Raisins	0.708	0.031
Butter	0.014	(0.22)†	Tapioca	0.020	0.004
Carrots	0.311	0.048	Walnuts	0.525	0.001
Cheese, hard	0.131	0.88†	Wheat, entire	0.465	0.006

* Information from Chemistry of Food and Nutrition, Henry C. Sherman, 7th ed., The Macmillan Co., New York, 1949. Reprinted by permission of the Macmillan Company.

† Data enclosed in parentheses are based on evidence either less consistent or less direct than in the majority of cases.

‡ Reports too discordant to average.

§ Uncertain because of varying methods of breadmaking.

¶ Varies with the amount of added salt.

[43] Violet Ashkins, and R. L. Zwemer, *J. Am. Diet. Assoc.* 14, 153 (1938).

Isotope studies *in vitro*, on specialized cells in an experimentally controlled environment, have yielded interesting data on factors affecting potassium accumulation by cells [37] Fertilized echinoderm eggs take up much larger amounts of K^{42} from artificial sea water than unfertilized eggs [38] Squid nerve sections will rapidly exchange about 10% of their potassium as indicated by the radioactive isotope in artificial sea water [39] The *rest of the potassium is only slowly* exchangeable. *This recalls the work on* measuring total body water with "heavy water," where nearly 24 hr. is needed for a reasonably uniform distribution.

From these and a variety of other experiments it would appear that not all the potassium in cells is in the same state of chemical or physical combination It is also clear that, since cell membranes are permeable to the cations sodium and potassium, one must assume that some active metabolic process is involved in maintaining the sometimes twentyfold differences in concentration between the inside and outside of living cells

There is evidence to indicate that oxygen, glucose, and temperature are all concerned with potassium accumulation Hemoglobin and myosin solutions can also concentrate potassium several times, when dialyzed against a semipermeable membrane This suggests that potassium can be accumulated directly or indirectly by living cells It is well to note that in red blood cells this can occur almost as well when sodium is replaced by lithium or cesium in the external environment [40] Simple fresh-water forms of life are also able to accumulate potassium even though the external medium is low in both sodium and potassium. The lower species of animals probably obtain most of theirs from ingested plant food, but the plants make a differential selection with retention of potassium and exclusion of sodium

An alternate theory of potassium accumulation in muscle suggests that it is the result of a double Donnan effect.[41, 42] Non-penetrating colloidal anions inside the muscle fiber set up an electrical potential gradient The positive sodium ions are actively extruded, and the potassium is accumulated by combination with the colloidal anion Both active extrusion of sodium and metabolic concentration of potassium may occur. The incorporation of potassium into large molecules might then hold both the cation and the necessary amount of water to maintain the iso-osmotic pressure within the cell Both water and cation would be released when the large molecule was catabolized.

[37] C W Sheppard, *Science* **114**, 85 (1951)

[38] E L Chambers, W White, Nylan Jeung, and S C Brooks, *Biol Bull* **95**, 252 (1948)

[39] M A Rothenberg, *Biochim et Biophys Acta* **4**, 96 (1950)

[40] E Ponder, *J. Gen Physiol* **33**, 745 (1950)

[41] E J Conway, *Irish J Med Sci* **6**, 593 (1947)

[42] E J Conway, *Biol Revs Cambridge Phil Soc* **20**, 56 (1945)

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Apples	0 116	0 002	Ham, medium to lean	0 383	¶
Bananas	0 373	0 002	Lamb (mutton)	0 301	0 084
Beans, dried	1 201	‡	Milk (cow's)	0 143	0 051
Beans, snap or string	0 251	0 003	Oysters	0 204	0 471
Beef, lean	0 338	0 084	Potatoes	0 496	0 005
Bread, white	0 100	0 446	Prunes, dry	0 848	0 008
Bread, whole wheat	(0 45)†	§	Raisins	0 708	0 031
Butter	0 014	(0 22)†	Tapioca	0 020	0 004
Carrots	0 311	0 048	Walnuts	0.525	0 004
Cheese, hard	0 131	0 88†	Wheat, entire	0 465	0 006

[illegible]

[43] Violet Ashkins, and R L Zwemer, *J Am Diet Assoc* **14**, 183 (1938)

The potassium content of food can be changed by methods of preparation or preservation. Some of these lower the potassium content, such as boiling in large quantities of water or repeated crystallization of the product as with cane or beet sugar. Other methods which remove water by heat or vacuum may increase the potassium manyfold. Reconstitution of the dried product with sufficient water will restore the original potassium ratio, but dried foods are frequently eaten in that form, with the high potassium content not generally recognized. Strong infusions of tea, and some soups, may also be rich in potassium.

The body as a whole may gain or lose potassium without gross changes in physiology. Difficulties arise, however, when the normal proportion of intracellular to extracellular potassium is altered by cell loss or plasma and tissue fluid gain of this element.[44]

3. Sodium in Extracellular Fluids

The distribution of sodium as the principle cation in extracellular fluid should not obscure the fact that, even though tissue sodium may be high by reason of the interstitial fluid present, some sodium must be present in cells. If one assumes 320 mg. per cent as the sodium content of extracellular fluid, then there would be about 9 g. in plasma and 31 g. in the interstitial fluids This with the reported value of 19 g in the skeleton would give a total of 59 g of sodium. The bone sodium is apparently labile and has been suggested as a possible reserve source of sodium at some stage of depletion of the extracellular body fluids.[45] Total sodium chloride in the body has been given as 175 g , which would be about 68 g. of sodium. This would suggest that about 9 g. of the sodium might be in cells.

The use of radioactive sodium (Na^{24}) has shown that *intracellular sodium* is diffusible and can pass freely through cells walls,[46] and labeled sodium *appeared in the pancreatic juice of dogs within 3 min. of intravenous injection.*[47] This confirms other studies which concluded that cell walls are permeable to free ions. The walls are conductors of direct current, even [illegible]

passing through epithelial cells of the skin. [illegible] sium content [48] The transport of sodium ions across cell membranes seems to require specific chemical reactions between inorganic ions and organic

44 [illegible] 649 (1950)

45 [illegible] t, R C Fowler, [illegible] 321 (1943)

46 [illegible]).

47 [illegible] Physiol 131, 578 1940

48 A. Krogh, *Skand. Arch. Physiol.* 76, 60 (1937)

cell constituents for metabolic transport against a gradient. This must be clearly distinguished from the diffusion of ions, if the membrane is freely permeable[49] Only the freely exchangeable and not the total sodium will be determined by the use of the radioactive isotope,[50] since some sodium is in an inactive state

The return of sodium and water to the plasma from glomerular filtrate would require a considerable osmotic effect of plasma protein with added metabolic transfer or exclusion of selected ions. To these sources of energy one may be able to add a new propulsive force for the filtrate. It has been suggested that the elastic double wall of the glomerular capsule may act as a hydraulic ram[51] It can be seen that there are a number of ways in which sodium and other ions may be moved from one intercellular space to another, or between extracellular and intracellular fluids

The complexity of the salt and water relationships are often obscured by the efficiency of the mechanisms for maintaining their constancy in the body, so that they do not seem to present nutritional problems

4 Sodium and Chloride in Nutrition

Vegetables and meat are all comparatively low in sodium, but no human dietary is so low that it cannot support life under conditions of normal activity Sodium can be spared by the excretion of fixed acids as potassium salts, or by the fixed base sparing mechanism of ammonia formation Herbivora and human beings accept a sodium chloride supplement when available A liberal daily allowance for an adult human would be 5 g, except for individuals who sweat profusely This with sodium chloride in food would give an average normal intake of 10 to 15 g and would be ample for a total water intake of 4 l When loss of fluid induces a greater water intake, then an additional gram of NaCl for each liter of water should be taken[52]

There are a number of conditions under which water, salt, or both may be lost from the body They include various types of stress, operative procedures, endocrine disturbances, and disease In some special cases the loss of sodium or chloride may predominate Marriott[15] has pointed out that the main differences between water depletion and salt depletion effects are related to the extracellular fluid osmotic pressure This becomes hypertonic with water loss and draws water from cells, producing marked thirst. In salt depletion the extracellular fluid is hypotonic, the kidneys, to maintain isotonicity, excrete water, and the volume of extracellular fluid is lowered.

[49] H H Ussing, *Physiol Revs* 29, 127 (1949)
[50] G B Forbes, and A Perley, *J Clin Invest* 30, 558 (1951)
[51] H H Zinsser, *Science* 114, 504 (1951)
[52] Recommended dietary allowances, National Research Council, Reprint and Circular Series No 129, Washington, D C, 1948

Since no water is withdrawn from cells, the sensation of thirst may be low or absent

In parenteral replacement it should be remembered that 0.7 l of isotonic saline will contain about 6 g. of salt and that this is about 10% more sodium and 50% more chlorine than is present in plasma. By careful calculation of both replacement needs and maintenance needs it should be possible to avoid undersalting in the replacement phase and oversalting in the maintenance phase of a dietary regimen [53]

As mentioned earlier, there are conditions, as in patients with acidosis and depressed renal function, when chloride should be reduced in proportion to sodium In these cases the lactate, which can be oxidized, or the bicarbonate, which can be excreted by the lungs, should replace the chloride.

Water and salt, familiar as they are in everyday experience, are being recognized more and more as dynamic participants in the living process.

[53] H Pollack, and S L Halpern, Therapeutic Nutrition, National Academy of Sciences, National Research Council, Washington, D C, 1951

CHAPTER 4

Carbohydrate Metabolism

T L SOURKES

Merck Institute for Therapeutic Research, Rahway, New Jersey

I. Introduction

Carbohydrates occupy a central role in the diet of man and animals In various parts of the world the mainstay of human nutrition is represented by rice, wheat, rye, maize, barley, oats, and potatoes, plants which contribute a cheap, easily digested and absorbed form of fuel for bodily energy requirements About 50% of the "average" American diet is made up of carbohydrate; this proportion is even higher in many countries where the more expensive proteinaceous foods and dietary fats are not as available

In contrast to plant foods, animal products other than milk make an insignificant contribution to the dietary supply of carbohydrate, since edible meats contain a negligible amount In spite of this, carbohydrates exhibit a high rate of turnover in animal metabolism There is no carbohydrate requirement in the sense applied to "essential amino acid" requirements, for the various carbohydrates and carbohydrate derivatives of the body may derive from non-carbohydrate sources. This has become abundantly clear from recent research in intermediary metabolism Protein, fat, and carbohydrate, or rather their digestion products, follow metabolic pathways some of which have now been shown to meet at common points In these "metabolic pools" the molecules lose their identity as to original source, and, since many of the pathways are reversible, the organic moieties undergo a more or less random distribution along the pathways leading away from the "pool "

II. The Role of Carbohydrates in Nutrition

1 The Common Dietary Carbohydrates

Carbohydrates make up the largest part by weight of the diet of economic animals and most humans and are the chief body fuel Protein and fat also contribute calories, of course, but they are depended upon to subserve other functions primarily The wide variety of carbohydrates and allied compounds occurring in natural foods do not lend themselves to routine analysis in the chemical laboratory, it is highly doubtful, in any case, whether such extensive analysis would provide useful nutritional information in proportion to the effort expended.

Dietary carbohydrate becomes available to the animal organism following breakdown to the monosaccharide stage The enzymes accomplishing these hydrolyses will be discussed in Chapter 17 and will not be dealt with here However, it must be mentioned that the enzymic apparatus deriving from both animal tissues and the intestinal microorganisms for the hydrolysis of the complex carbohydrates is not completely efficient; that is to say, "available carbohydrate" is always less than "carbohydrate as consumed" in natural mixed diets This fact has led to the distinction between two broadly characterized categories: a "poorly" digested and a "highly" digested fraction To the latter belong starch, glycogen, and sugars, together with much of the hemicellulose The low digestibility fraction includes the complex polysaccharides of the plant cell wall

A brief description of some of the cell wall constituents will be found useful in the discussion which follows *Cellulose* ("true" cellulose), chemically speaking, is a polymer of glucose It forms interpenetrating systems (but not chemical linkages) with hemicellulose and lignin,[1] but it may also occur independently of these The portion of the cell wall which is soluble in alkali and hydrolyzable by dilute acid constitutes the *hemicelluloses* This term was coined by Schultze in 1891 to include those cell wall components that are intermediate in solubility and other properties between storage carbohydrates (like starch) and the structural polysaccharides such as cellulose The ultimate products of hydrolysis are largely xylose, uronic acid units, acetic acid, and small amounts of glucose and mannose *"Pectic substances"* are "those complex, colloidal carbohydrate derivatives which occur in or are prepared from plants and contain a large proportion of anhydrogalacturonic acid units which are thought to exist in chain-like combination The carboxyl groups of the polygalacturonic acids may be partly esterified by methyl groups and partly or completely neutralized by one or more bases"[2] In the plant cell wall pectic substances occur as deposits in association with cellulose The base is usually calcium, although sometimes magnesium is present The term *cellulosan* was proposed[3] to cover polymers of hexoses or pentoses which occur in nature intermixed with "true" cellulose. These

are chiefly xylan, but some glucosan may also be found Although not a carbohydrate, *lignin* is so closely associated with the carbohydrates of the cell wall that it merits discussion here It is essentially an aromatic substance related to the coniferyl type of compounds and is of high molecular weight. Its close association with cellulose chains has led some to refer to a "lignocellulose" fraction See review by Percival[3a]

Because of the inherent difficulties in analyzing for dietary carbohydrates it has been customary to determine them as a group by subtracting from the dry weight of the diet the crude protein (nitrogen × 6 25), ether extract, and ash, the difference representing "total carbohydrate." In this scheme of "proximate analysis" all the analytical errors are included in this remaining fraction A refinement, the value of which has been frequently questioned, was introduced in 1864 by Henneberg and Stohmann at the Weende Agricultural Experiment Station in Germany. This involved determining the "crude fiber," i e , the fraction consisting of organic matter insoluble in water, ether, and alcohol, which remains after successive refluxing with 1 25% sulfuric acid and 1.25% sodium hydroxide The portion of the sample remaining after deductions for moisture, protein, ether extract, crude fiber, and ash is known as the nitrogen-free extract. Henneberg recognized the deficiences of his method- ical composition of the isolated "fiber"- give an approximate determination of method, somewhat modified, is still widely used and, indeed, is an official procedure in the Association of Official Agricultural Chemists[4]

There is adequate justification for seeking analytical categories which correspond to biological units of digestibility, for routine analyses would then free the nutritionist from the prolonged animal feeding trials necessary for digestibility determinations and would at the same time give him information of value in planning dietary regimes However, the inadequacy of the crude fiber/nitrogen-free extract partition has often been the target of criticism For example, it has been shown that crude fiber is frequently as digestible as the nitrogen-free extract[5] Furthermore, although most evidence points to the complete non-digestibility of lignin even in ruminants,[5, 6] Norman found[7] that in the crude fiber determination most of the lignin ends up in the nitrogen-free extract, i e , in the supposedly easily digested fraction This was also the experience of Remy,[8] who separated the cellulose-hemicellulose-lignin group from the starch, protein, and fat by enzymatic means, the former was twice as great as crude fiber pre-

[3a] E G V Percival, *Brit J Nutrition* 6, 104 (1952)

[4] Association of Official Agricultural Chemists, Official Methods of Analysis 7th ed , *Washington, D C* , 1950

[5]

[6]

[7]

[8]

pared by the more drastic Weende procedure Mangold has discussed the digestion and utilization of crude fiber in many species [9]

Recognizing the limitations of the conventional crude fiber determination, various workers have advocated more direct methods of estimating available carbohydrate, employing hydrolysis of the food constituents by means of enzymes such as those contained in takadiastase and pancreatin (Williams and Olmsted have reviewed the older literature in this field [10]) Others have suggested analytical procedures to include more specific categories McCance and Widdowson[11] determined "available carbohydrate" as the directly determined starch, sugars, and dextrins, thus obviating analysis of the "poorly digestible" components Williams and Olmstead[10] determined lignin, hemicellulose, and cellulose Crampton and Maynard[6] proposed the partition into cellulose, lignin, and "other carbohydrates" Methods were presented for the first two, "other carbohydrates" being determined by difference Evidence was adduced to illustrate a greater biological significance for these units and their usefulness in predicting feeding values Because of the difficulties encountered in the lignin determination a modified scheme was later presented,[12] but recent methods for lignin[6] and cellulose[13] may help resolve some of these problems

In regard to the human diet, which generally contains little fiber (about 5 to 20 g per day), carbohydrate digestibility is very high From his extensive studies made at the end of the last century, Atwater arrived at the figure of 97% for the digestibility coefficient of carbohydrate in "the average American diet "[14] This is still considered valid,[15] but it does not apply to diets containing larger amounts of coarse foods Nor can it be used when individual foods are considered, for these vary in carbohydrate digestibility: meats, 98%, cereals, 98%, legumes, 97%, vegetables, 95%, fruits, 90%, and sugars, 98% Moreover, Atwater's average has been weighted for the proportions of each food group occurring in the diets he examined

The problem takes on greater significance for the feeding of economic animals, in that their diets contain large proportions of plant foods with correspondingly high amounts of indigestible cell wall constituents These may not only "dilute" the feed but, if the cell wall is not made permeable, may also carry through the intestinal tract some of the enclosed digestible nutrients On the other hand the fiber prevents "packing" of the food, provides additional bulk for normal laxation, and also serves as substrate for the intestinal microorganisms

Further losses from the ingested carbohydrate fraction are encountered

[9] E Mangold, *Nutrition Abstracts & Revs* **3**, 647 (1934)

[10] R D Williams and W H Olmsted, *J Biol Chem* **108**, 652 (1935)

[11] R A McCance and E M Widdowson, The Chemical Composition of Foods, Medical Research Council, Special Report 235, London, 1940.

[12] E W Crampton and F Whiting, *J Animal Sci* **2**, 278 (1942)

[13] G Matrone, G H Ellis, and L A Maynard, *J Animal Sci* **5**, 306 (1946)

[14] W O Atwater, *U S Dept Agr. Expt Sta Bull* **21** (1895)

[15] L A Maynard, *J Nutrition* **28**, 443 (1944)

owing to gas formation (CO_2, CH_4, H_2) by the intestinal bacteria, especially in ruminants and horses.[16] Various fermentation acids (acetic, lactic, butyric, isobutyric, valeric, formic, propionic, and succinic) are also produced and contribute to laxation. A portion of these, however, are absorbed and utilized. The digestibility of crude fiber is depressed by the presence of large amounts of sugars in the ration, as has been demonstrated in calves[17] and sheep.[18, 19] This is attributed to the preference by the microorganisms of the paunch for the sugar. The bacterial degradation of starches in the digestive tract of ruminants and non-ruminants has been compared by Baker *et al*.[20] The converse effect of various dietary carbohydrates on the intestinal flora has been reviewed by Johansson and Sarles.[21]

2. Absorption of Ingested Carbohydrates

Before absorption, carbohydrates must be degraded to the monosaccharide stage, unhydrolyzed polysaccharides are not absorbed Among the hydrolytic products of the carbohydrate portion of natural diets, glucose is by far the most abundant sugar, and galactose and fructose make up most of the remainder. Absorption occurs mainly in the small intestine where a gradient exists, with the highest rate in the upper portion. Little or no sugar is absorbed from the rectum.[22]

Under carefully circumscribed experimental conditions the rate of absorption of glucose from the intestinal tract appears to be independent of the amount of sugar in the intestinal lumen This has been demonstrated in the rat[23] and the dog.[24] In such experiments, galactose is absorbed more rapidly than glucose, fructose, mannose, L-xylose, and L-arabinose are absorbed at slower rates, in decreasing order as listed.[23, 25] However, under "normal" conditions, or in voluntary feeding, the rate of absorption may be higher than that usually found in gavage experiments[26] and, in fact, is affected by the nature of the other substances in the intestine at the

16 H H. Mitchell, *J. Animal Sci.* **1**, 159 (1942).
17 H. H. Mitchell and T. S. Hamilton, *J. Agr. Research* **61**, 847 (1940).
18 T S. Hamilton, *J Nutrition* **23**, 101 (1942)
19 R. W Swift, E J. Thacker, A. Black, J W Bratzler, and W H. James, *J Animal Sci.* **6**, 432 (1947)
20 F. Baker, H. Nasr, F. Morrice, and J Bruce, *J. Path Bact* **62**, 617 (1950).
21 K. R. Johansson and W. B. Sarles, *Bact. Revs* **13**, 25 (1949).
22 J. P Peters and D D. Van Slyke, Quantitative Clinical Chemistry, Vol. I, 2nd ed , The Williams and Wilkins Co , Baltimore, 1946.
23 C F. Cori, *J. Biol. Chem.* **66**, 691 (1925).
24 H. C Trimble, B W. Carey, Jr , and S J. Maddock, *J. Biol Chem* **100**, 125

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time, by the state of salt and water metabolism of the individual, by hormonal factors, and by other conditions

The mechanism of absorption from the intestinal tract involves a concentration gradient between the sugar in the intestinal lumen and that inside the cells of the mucous membrane Diffusion under the influence of such a gradient is probably accelerated by phosphorylation of the sugars (at least the hexoses) within the cells That phosphorylation occurs is reflected in the increase in organic phosphate in the intestinal mucosa during the period of sugar absorption [27, 28] Furthermore, poisons, such as phlorizin[23] and iodoacetic acid,[25] which interfere with phosphorylation processes, also inhibit hexose absorption Absorption of pentoses is not affected by iodoacetic acid [25]

3 Parenteral Administration of Carbohydrates

Parenteral administration of glucose has become routine in many clinical and experimental procedures Since the intestine is by-passed, digestibility is 100% efficient, 1 g of glucose yielding the theoretical 3 76 cal To avoid glucosuria the rate of administration must not be greater than about 0 5 to 0 75 g per kilogram body weight per hour,[29] consequently provision of adequate calories by this route calls for the use of prolonged administration of large volumes of fluid The rate of fructose utilization by intravenous injection is, however, considerably greater [29a]

The search for cheap and readily available blood and plasma substitutes for use in shock and surgery has occasionally turned to polysaccharides because of their colloidal properties in solution Neutral gum arabic solutions (6 to 7% in physiological saline) were introduced by Bayliss at the time of the first world war These provided a satisfactory immediate response but had little effect in reducing mortality rates and so lost favor. Pectin solutions have also been found to be effective in maintaining the blood pressure in experimental animals suffering shock, but pathological changes may occur following their use The use of glycogen has occasionally been suggested Intravenously administered glycogen remains in the plasma for long periods and is only slowly broken down to dextrins and

[27] L Laszt and H Sullman, *Biochem Z* **278**, 401 (1935), K Kjerulf-Jensen, *Acta Physiol Scand* **4**, 225, 249 (1942)

[28] L V Beck, *J Biol Chem* **143**, 402 (1942)

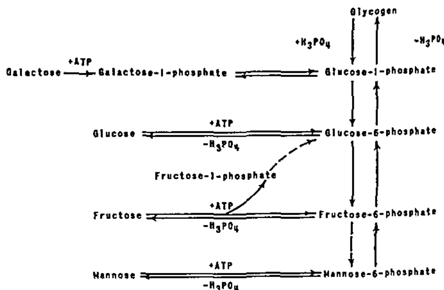
[29] T. B Van Itallie, *Nutrition Revs* **9**, 193 (1951)

[29a] T E Weichselbaum, R Elman, and R H Lund, *Proc Soc Exptl. Biol. Med.* **75**, 816 (1950), J J Weinstein, *J Lab Clin Med* **38**, 70 (1951), Editorial, *J. Am Med Assn* **145**, 987 (1951), F Lasch and H Kaloud, *Deut. med. Wochschr.* **76**, 895 (1951)

glucose[30] A promising new material, the bacterial polysaccharide dextran, is currently being investigated and evaluated.[31]

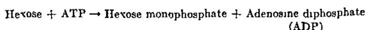
4 Assimilation of Dietary Hexoses: Glycogen Formation (Scheme 1)

a. Initial Phosphorylation of Sugars. Having passed the intestinal barrier the dietary hexoses are carried in the blood stream to the liver and, eventually, to the other tissues where they are synthesized into glycogen The



Scheme 1. Enzymatic interconversions of the hexoses, and the formation of glycogen

initial step of this process is the phosphorylation of the sugar by adenosine triphosphate (ATP):[31a]



Hexose + ATP → Hexose monophosphate + Adenosine diphosphate (ADP)

Enzymes mediating reactions of this type are known as hexokinases It

[30] D L Morris, *J Biol Chem*, **148**, 699 (1943), W L Bloom, C J. Nichols, and J Busey, *Am J. Physiol* **165**, 288 (1951)

[31] A Grönwall and B Ingelman, *Nature* **155**, 45 (1945), Anon, *Brit Med J* 591 (1951)

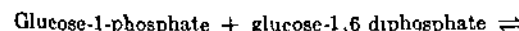
[31a]

thiamine (cocarboxylase)

has been suggested that mammalian tissues contain a non-specific hexokinase acting on glucose, fructose, and mannose, as well as a second, specific fructokinase [32] The product of glucose phosphorylation is glucose-6-phosphate In the case of fructose, a liver enzyme converts it to fructose-1-phosphate,[33] although the 6-phosphate may be formed in other tissues.

The mechanism of galactose assimilation has only recently received some clarification This sugar is phosphorylated by ATP in the 1 position, forming a phospho-acetal, as described by Trucco and co-workers [34] While this group purified "galactokinase" from the lactose-fermenting *Saccharomyces fragilis* and galactose-adapted brewer's yeast, the existence of such an enzyme in liver may well be inferred from Kosterlitz' finding several years ago that galactose-1-phosphate accumulates in the liver of rabbits fed galactose,[35] and, in fact, identification of this enzyme in liver has been claimed [36]

b. Isomerization of Glucose-6-phosphate. Glucose-6-phosphate undergoes reversible isomerization to glucose-1-phosphate[37] through the influence of phosphoglucomutase, an enzyme found in animal tissues as well as in yeast [38] Phosphoglucomutase has no action on galactose- or mannose-1-phosphate [39] The equilibrium is well in favor of the 6-ester


Glucose-1-phosphate + glucose-1,6 diphosphate ⇌


Glucose 1,6-diphosphate + glucose-6-phosphate

In 1938, Kendal and Stickland[40] provided evidence that the mutase is activated by fructose-1,6-diphosphate Actually, the coenzyme responsible for activation is glucose-1,6-diphosphate,[41] a compound occurring as a contaminant in preparations of the fructose diester Kendal and Stickland admittedly used an impure salt of fructose-1,6-diphosphate in their work Leloir and co-workers have synthesized the coenzyme of phosphogluco-

[32] S P Colowick, *in* Sumner and Myrback, The Enzymes, Academic Press, New York, 1951, Vol 2, Part 1, Chapter 46, F Leuthardt and E Testa, *Helv Chim Acta* **33**, 1919 (1950)

[33] G T Cori, S Ochoa, M W Slein, and C F Cori, *Biochim et Biophys Acta* **7**, 304 (1951), A Staub and C S Vestling, *J Biol Chem* **191**, 395 (1951), H G Hers, *Biochim et Biophys Acta* **8**, 416 (1952)

[34] R E Trucco, R Caputto, L F Leloir, and N Mittelman, *Arch Biochem* **18**, 137 (1948).

[35] H W Kosterlitz, *Biochem J* **31**, 2217 (1937), *ibid* **37**, 318 (1943)

[36] M Becila, *Arquiv biol e ternol , Inst biol e pesquisas tecnol , Curitiba, Brasil* **3**, 3 (1948) [C A **44**, 5942 (1950)]

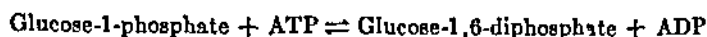
[37] E W Sutherland, S P Colowick, and C F. Cori, *J Biol Chem* **140**, 309 (1941)

[38] G T Cori, S P Colowick, and C F Cori, *J Biol Chem* **123**, 375 (1938)

[39] G T Cori, S P Colowick, and C F Cori, *J Biol Chem* **124**, 543 (1938)

[40] L P Kendall and L H Stickland, *Biochem J* **32**, 572 (1938)

mutase using purified yeast[41] or muscle[42] preparations:



Glucose-1-phosphate + ATP ⇌ Glucose-1,6-diphosphate + ADP

Phosphoglucomutase has been crystallized from muscle.[43] The enzyme constitutes 2% of muscle protein.

c. Isomerization of Galactose-1-phosphate. Galactose-1-phosphate, the Kosterlitz ester, is isomerized to glucose-1-phosphate by inversion of its carbon atom number 4.[44] Caputto *et al.*, who discovered this interesting reaction, assigned to the enzyme involved the title "galactowaldenase." They also described a coenzyme for galactowaldenase, namely uridine diphosphate glucose. The coenzyme is found in baker's yeast and, in smaller amounts, in animal tissues; on treatment with yeast extract it forms some uridine diphosphate galactose.[45]

Thus, galactose can form glycogen by passing through the galactose-1-phosphate and glucose-1-phosphate stages Even on continued galactose feeding, muscle and liver glycogen contain no galactose units,[46] although a galactogen has been found in preparations of crude heparin from beef lung.[47]

d. Isomerization of Fructose-6-phosphate and Mannose-6-phosphate. In 1933 Lohmann demonstrated the reversible conversion of glucose-6-phosphate to fructose-6-phosphate in preparations of frog tissues.[48] (The equilibrium mixture of the two sugar phosphates was known as "the Embden ester," before its dual nature was recognized.) Another isomerase from rabbit muscle can convert the 6-phosphate ester of glucose, fructose, or mannose to an equilibrium mixture of all three[49] In this way, fructose and mannose are glycogen formers by virtue of the conversion of their phosphorylated derivatives to glucose-6-phosphate. Fructose-1-phosphate is also converted to this ester by way of as yet unknown intermediates[33]

e. Phosphorylase. In 1935–1936 Parnas and Ostern showed that glucose-6-phosphate accumulates when glycogen and inorganic phosphate are incubated with dialyzed muscle extract. It remained for the Coris to show

[41] L. F Leloir, R E Trucco, C E Cardini, A. Paladini, and R Caputto, *Arch Biochem* **19**, 339 (1948)

[42] A Paladini, R Caputto, L F Leloir, R. E. Trucco, and E. Cardini, *Arch Biochem* **23**, 55 (1949)

[43] V A Najjar, *J Biol Chem* **175**, 281 (1948).

[44] R Caputto, L F Leloir, C E Cardini, A. C Paladini, *J. Biol Chem* **184**, 333 (1950), Y J Topper and D Stetten, Jr, *J. Biol Chem* **193**, 149 (1951).

[45] L. F Leloir, *Arch Biochem Biophys* **33**, 186 (1951), A C Paladini and L F. Leloir, *Biochem J.* **51**, 426 (1952)

[46] V. J. Harding, G A. Grant, and D. Glaister, *Biochem J.* **28**, 257 (1934).

[47] M. L Wolfrom, D. I. Weisblat, J V. Karabinos, and O Keller, *Arch Biochem* **14**, 1 (1947)

[48] K. Lohmann, *Biochem Z* **262**, 137 (1933)

[49] M. W Slein, *J. Biol Chem.* **186**, 753 (1950)

that the first product of this phosphorolysis was a new, non-reducing glu ester, identified as glucose-1-phosphate by isolation and synthesis [50] Br liver, muscle, and heart preparations catalyze the phosphorolysis, but extracts must be dialyzed to remove magnesium ions These permit action of phosphoglucomutase, which converts the 1-ester to glucos phosphate,[35] and in their absence the former compound is obtained. T phosphorylyzing type of enzyme, known as phosphorylase, also occur yeast, potatoes, peas and certain bacteria, as well as other sources. sides glycogen, amylopectin (the branched component of starch) can se as substrates In both cases, depolymerization occurs beyond the point branching

The phosphorolysis reaction is reversible[38, 51, 52] since glucose-1-pl phosphate is transformed by the action of phosphorylase into a poly charide with the release of inorganic phosphate ("dephosphorolysi For the synthetic reaction to take place, a small amount of prefor polysaccharide (starch, glycogen, dextrin) must be present as a "prim In this case, heart and liver phosphorylases form a glycogen-like poly charide (i e, with branching chains), but the crystalline muscle enz forms a linear chain resembling the amylose component of starch Si muscle contains no amylose-like polysaccharide, it appears that the p merizing action of crystalline muscle phosphorylase is supplemented in intact tissue by another enzyme to yield glycogen A heat-labile fractio heart and liver[53] or of muscle[54] can serve as such a "branching factor' *vitro* for, if it is combined with muscle phosphorylase, a glycogen-like p saccharide is formed from glucose-1-phosphate Thus, glycogen synth *in vivo* may be regarded as the result of action of two phosphoryla effecting α-1,4 linkages and α-1,6 linkages, respectively, between glucosidic residues of glucose-1-phosphate.[53]

5 Formation of Blood Sugar

Until the phosphorolysis of glycogen was demonstrated it had h generally assumed that the blood sugar is formed from liver glycogen an amylase type of action (i e, hydrolysis), in spite of the fact that amyl occurring in animals yield mainly intermediate hydrolytic products are hydrolyzed to glucose only slowly It is now clear that when glyco is broken down intracellularly there occurs essentially a reversal of

[50] C F Cori, S P Colowick, and G T Cori, *J Biol Chem* **121**, 465 (1937) T Cori and C F Cori, *Proc Soc Exptl Biol Med* **36**, 119 (1937)

[51] G T Cori, C F Cori, and G Schmidt, *J Biol Chem*, **129**, 629 (1939), Kiessling, *Biochem Z* **302**, 50 (1939)

[52] C S Hanes, *Proc Roy Soc (London)* **B129**, 174 (1940).

[53] G T Cori and C F Cori, *J Biol Chem* **151**, 57 (1943)

[54] A N Petrova, *Biokhimiya* **13**, 244 (1948), **17**, 129 (1952)

synthesis from glucose. However, the hexokinase reaction is irreversible, since the ester phosphate of the hexose phosphates cannot phosphorylate ADP The role of hydrolyzing this linkage is played by glucose-6-phosphatase, an enzyme found in the liver.[55]

6 Formation of Fetal Blood Sugar and Seminal Plasma Fructose

Much free fructose is found in the fetal blood,[55a] in amniotic fluid,[56] and in seminal plasma [57] Mann and Lutwak-Mann[58] have found a phosphohexose isomerase in bull seminal vesicles which could account for the formation of fructose-6-phosphate from the corresponding glucose ester Since this organ may contain up to 1% of glycogen, there is a readily available source of the monosaccharides, besides the blood sugar. Presumably a fructose-6-phosphatase also occurs in the seminal vesicles.

A phosphohexose isomerase has been detected in placental tissue, as well as an alkaline phosphatase, acting on fructose-6-phosphate.[59] These enzymes can then account for the origin of the fetal blood fructose.

7 Amount of Carbohydrate in the Diet

Carbohydrate is ordinarily an abundant ingredient of the human diet, no minimum requirement has been established, since carbohydrates can be synthesized in the body from other dietary constituents Moreover, the latter can satisfy the energy requirements, if consumed in sufficient amount. Nevertheless, some nutritionists have expressed the opinion that carbohydrate should supply at least 15 to 20% of the energy in a balanced diet The Food and Nutrition Board of the National Research Council[60] recommends a dietary allowance of 70 g of protein per day for the "average" adult male (70 kg), with about 20 to 25% of the total allowance of 3000 cal to come from fat By implication this gives an estimate of about 500 g of carbohydrate per day

8 Energetic Considerations

Various carbohydrates are not equivalent in respect to the amount of energy available from their complete combustion For example, as deter-

[55] R H Broh-Kahn, I A Mirsky, G Perisutti, and J Brand, *Arch Biochem* 16, 87 (1948), C de Duve, J Berthet, H G Hers, and L Dupret, *Bull soc chim biol* 31, 1242 (1949), H G Hers, J Berthet, L Berthet, and C de Duve, *Bull soc chim biol* 33, 21 (1951)

[illegible]

mined in the bomb calorimeter starches have a greater caloric yield per gram than the simple sugars (glucose, 3 76 cal per gram) The heat of hydrolysis of the polysaccharides in the intestinal tract contributes only to heat production and is not available for work. Because some food carbohydrate escapes digestion and absorption from the intestine, a correspondingly small proportion of the energy present in the carbohydrate fraction of the diet is actually not available In his studies on American diets Atwater[14] took these digestion losses into account in arriving at the figure of 4 cal per gram for the "physiological fuel value" of carbohydrate As pointed out before in connection with the digestibility coefficient of carbohydrates, this is a weighted average which holds for diets similar to those Atwater studied but which would not apply, and should not be applied uncritically, to diets containing more roughage or less, as in many diets containing purified nutrients used in animal experiments Likewise when a single carbohydrate is used in such diets the physiological fuel value requires correction

Further losses in energy derived from carbohydrate are encountered in metabolism, although they are ordinarily small Following ingestion of food under otherwise basal metabolic conditions there is a small increase in the metabolic rate, termed "the specific dynamic action" of the food The early view that the specific dynamic action is a constant proportion of the consumed calories (5% in the case of carbohydrate) has persisted for many years But the significance of the dynamic effects of individual foods as biological constants has been challenged by Forbes and co-workers They have demonstrated[51] that the heat increment, or "energy expense of utilization," is affected by the nutrient combination, the disposition of the absorbed nutrients (i e , the use to which they are put, whether growth, maintenance, work, etc) and the plane of nutrition prior to the experiment

9. Excretion of Sugar in the Urine

Excretion of glucose in the urine in very small amounts appears to be a normal process These concentrations are less than 0 10%, the level of sensitivity of the usual qualitative tests used in the clinical laboratory Such small amounts of reducing substances, even if estimated entirely as glucose, are unimportant for the energy economy of the body However, the larger amounts which occur in the urine of diabetic individuals may represent substantial losses Glucosuria is occasionally seen in some otherwise healthy individuals Pentosuria is attributed to a defect in metabolism but is not associated with a clinical state

[51] E B Forbes and R W Swift, *J Nutrition* **27**, 453 (1944), E. B Forbes, R. W Swift, R F Elliott, and W H James, *J. Nutrition* **31**, 213 (1946)

III. Intermediary Metabolism

1. Historical

Sugar fermentation by yeast, one of the oldest of the "biochemical arts," has interested chemists for centuries. The French chemists, from Lavoisier on, attempted to study fermentation quantitatively. Thus, Gay-Lussac *succeeded in showing that the grape sugar fermented in his experiments* could be accounted for by the alcohol and carbon dioxide produced (aside from lesser fermentation products). In spite of the demonstration of this "chemical balance sheet," clarification of the intermediary steps made little or no headway during the nineteenth century. The discovery of yeast as a vegetative organism in the 1830's was not at first generally accepted and, in fact, was met with derision and scorn by some of the leading chemists of that day including Berzelius, Wohler, and Liebig. The opposition abated after Pasteur presented his imposing researches on fermentations by yeast and lactic acid bacteria The failure to dissociate fermentation from intact cells, however, and the limitations imposed upon the experimental study of intermediary processes using living cells formed the basis for the celebrated Liebig-Pasteur controversy over the mechanism of fermentation. The *matter was settled by the accidental discovery of sugar fermentation* in a cell-free preparation of yeast by Buchner in 1897. This finding presented the possibility of chemically dissecting the process into its consecutive steps Initial successes with cell-free yeast juice included the discovery of a coenzyme of fermentation, the catalytic action of inorganic phosphate on cell-free fermentation, and the formation of phosphorylated intermediates (Harden's monograph[62] contains an extensive review of the earlier literature as well as the historical development of investigations on alcoholic fermentation.)

A parallel problem confronted the physiologists, i.e., the breakdown of glycogen in animal tissues, a process which was known to occur during muscular contraction, and to yield lactic acid when muscle was made to contract anaerobically. Claude Bernard had studied the action of many tissues on glucose and noted the appearance of lactic acid as sugar disappeared. This "lactic fermentation" took on added significance when the later studies on cell-free fermentation of sugar began to appear. The increased glycolysis in contracting muscle compared with muscle at rest, the formation of lactic acid anaerobically, the isolation of phosphorylated compounds from the tissues—these and many other results indicated a marked similarity between the processes occurring in yeast (and certain bacteria) and in animal tissues. Another point of similarity was in the exhibition of the "Pasteur reaction": the inhibition of fermentation (alcohol

[62] A. Harden, Alcoholic Fermentation, 3rd ed , Longmans, Green and Co , London, 1923.

and lactic acid formation, respectively) in the presence of oxygen, and substitution of an oxidative type of metabolism The vast amount of work which has been done in these fields since the turn of the century has placed our knowledge of the intermediary metabolism of carbohydrates far ahead of our understanding of fat and protein metabolism and, indeed, of many other areas of biochemical investigation

2 Intermediary Metabolism of Glucose. Alternate Pathways

Several pathways for the breakdown of glucose have been proposed, but only one has been extensively explored, namely, the Meyerhof-Embden-Parnas scheme of glycolysis While glycolysis occurs aerobically in tumor and retinal tissues, and anaerobically in many others, no net oxygen uptake (or hydrogen loss) is registered in the conversion of 1 molecule of hexose to 2 molecules of lactic acid, so that this is termed the anaerobic pathway. The essential steps are the formation of hexose diphosphate, the breaking of the C_3-C_4 bond to yield triose phosphate, dehydrogenation of the latter to a glyceric acid derivative, and conversion of this compound to pyruvic acid which is finally reduced to lactic acid

In 1931–1938 experiments by Warburg, Lipmann, and Dickens suggested the existence of an oxidative pathway, in which the primary phosphorylative product of glucose, the 6-phosphate ester, is oxidized directly to 6-phosphogluconic acid A further oxidation at the α-carbon atom, followed by decarboxylation, would yield a pentose-5-phosphate By two repetitions of this process one would arrive at glyceraldehyde-3-phosphate, having lost 3 carbon atoms as carbon dioxide This scheme has been suggested also as a path of origin of ribose in the animal body, but a stumbling block to its acceptance has been the fact that decarboxylation of 2-keto-6-phosphogluconic results in arabinose-5-phosphate, and not the ribose ester

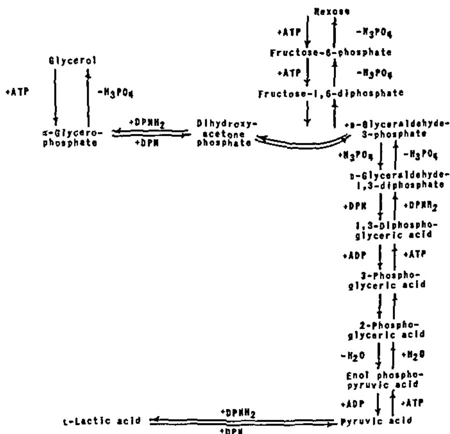
Lately, however, new enzymatic reactions have been described which indicate how pentose may be formed from 6-phosphogluconic acid, as well as from triose phosphate The reverse of the latter reaction, that is, triose phosphate formation from ribose, would then link this oxidative pathway with anaerobic glycolysis

Some of the reactions discussed in this section have thus far been demonstrated only in yeast or bacteria and therefore do not provide conclusive evidence that corresponding enzymes exist in animal tissues However, the development of the field of intermediary metabolism has made extensive use of the concept of analogy, and the results have shown it to be a useful one

3 Glycolysis (Scheme 2)

a. Anaerobic Formation of Trioses. The initial reactions of this pathway, namely phosphorylation of the hexoses and interconvertibility of their phosphates, have already been described (Sections II 4 and II 5).

Under conditions favoring glycolysis, these reactions funnel into fructose-6-phosphate, which is then phosphorylated once more by ATP to form fructose-1,6-diphosphate (the Harden-Young ester). Fructose-1-phosphate can also be phosphorylated to the same product by an analogous reaction,[63] but whether the same enzyme is involved is not known.



SCHEME 2 Meyerhof-Embden-Parnas scheme of glycolysis.

The origin of the 3-carbon compounds formed in the fermentation of

of the hexose chain into two 3-carbon molecules

Thus many compounds such as glyceraldehyde, glyceric acid, glycerol,

[63] G. T Cori and M W. Slein, *Federation Proc* 6, 245 (1947).

dihydroxyacetone, methyl glyoxal, triose phosphate, acetaldehyde, and formate, as well as a variety of C_4 and C_6 hydroxy acids, were implicated as intermediaries in the breakdown of glucose. The crude analogy between test-tube and enzymatic degradation of glucose neglected the directive role which the enzymes of glycolysis play in this process and left unexplained for many years the important role of the phosporylated derivatives of some of these intermediates. For example, twenty-five years separated the discovery in 1908 by Harden and Young of the formation of hexose diphosphate in the course of cell-free fermentation[64] and the demonstration by Meyerhof and Lohmann that this diester is the substrate for enzymes in muscle and yeast yielding triose phosphate.[65] The conversion occurs in two steps. (*a*) the splitting of fructose-1,6-diphosphate into D-glyceraldehyde-3-phosphate and dihydroxyacetone monophosphate, mediated by "aldolase,"[66] and (*b*) the conversion of the D-glyceraldehyde-3-phosphate to dihydroxyacetone phosphate by "triose phosphate isomerase."[67] The two enzymic components of zymohexase (the unpurified mixture) were separated a few years later by Herbert *et al.*[68] Aldolase was crystallized by Warburg and Christian[69] and Taylor *et al.*[70] Triose phosphate isomerase was purified by Meyerhof and Beck.[71] The action of zymohexase on fructose-1,6-diphosphate results in the appearance of mainly dihydroxyacetone phosphate,[65] owing to the rapid conversion of glyceraldehyde-3-phosphate to this compound. Only the D-isomer of the glyceraldehyde ester is fermented by yeast juice; in purified preparations, dihydroxyacetone phosphate is carried further along the path of glycolysis only if the triose phosphate isomerase is present.

The widespread occurrence of an enzyme, glyoxalase,[72] catalyzing the conversion of methyl glyoxal to lactic acid, was at one time considered justification for including methyl glyoxal as a probable intermediate in glycolysis. However, the lactic acid resulting from glyoxalase action is of the D configuration, whereas breakdown of glucose in tissues yields L-lactic acid. Furthermore, although glyoxalase required glutathione as coenzyme, Lohmann[73] was able to obtain glycolysis of glycogen in preparations con-

[64] A. Harden and W. J. Young, *Proc. Roy. Soc. (London)* **B80**, 299 (1908).
[65] O. Meyerhof and K. Lohmann, *Naturwissenschaften* **22**, 220 (1934); *Biochem. Z.* **271**, 89 (1934).
[66] O. Meyerhof, K. Lohmann, and P. Schuster, *Biochem. Z.* **286**, 301, 319 (1936).
[67] O. Meyerhof and W. Kiessling, *Biochem. Z.* **279**, 40 (1935).
[68] D. Herbert, H. Gordon, V. Subramanyan, and D. E. Green, *Biochem. J.* **34**, 1108 (1934).
[69] O. Warburg and W. Christian, *Biochem. Z.* **314**, 149 (1943).
[70] J. F. Taylor, A. A. Green, and G. T. Cori, *J. Biol. Chem.* **173**, 591 (1948).
[71] O. Meyerhof and L. V. Beck, *J. Biol. Chem.* **156**, 109 (1944).
[72] H. D. Dakin and H. W. Dudley, *J. Biol. Chem.* **14**, 155, 423 (1913).
[73] K. Lohmann, *Biochem. Z.* **254**, 332 (1932).

taining no detectable glutathione The methyl glyoxal which may appear in the course of glycolysis seems to come from fructose-1,6-diphosphate.[74]

Recently "glyoxalase" has been separated into two entities: the first brings about the formation of an addition compound between the substrate and glutathione, the second accelerates the decomposition of this compound into D-lactic acid and coenzyme.[75]

b. Reactions of the Triose Phosphates. As mentioned, glyceradehyde-3-phosphate and dihydroxyacetone phosphate can be condensed by aldolase to form fructose diphosphate. The reaction is apparently non-specific for the aldehyde partner, since a variety of simpler aldehydes can substitute for glyceraldehyde-3-phosphate.[66] The products of some of these condensations are: 4-methyl-2-ketotetrose-1-phosphate (dihydroxyacetone phosphate with acetaldehyde); D-fructose-1-phosphate (with D-glyceraldehyde, and L-sorbose-1-phosphate (with L-glyceraldehyde) The last-named product is probably responsible for the inhibition of glycolysis by DL-glyceraldehyde, since Lardy and his co-workers have shown that the L-sorbose ester is a strong inhibitor of hexokinase.[76] In suitable extracts of brain and liver tumor, tagatose-6-phosphate (the 2-ketose corresponding to galactose) forms the same two trioses as does fructose-1,6-diphosphate.[77]

Alcoholic fermentation is usually accompanied, at least in the early stages, by the appearance of small amounts of glycerol, owing to the reduction of dihydroxyacetone phosphate by reduced diphosphopyridine nucleotide (reduced coenzyme I, $DPNH_2$), and subsequent hydrolysis of the α-glycerophosphate so formed Ordinarily, acetaldehyde, formed by the decarboxylation of pyruvic acid in the course of yeast fermentation, serves to reoxidize the reduced coenzyme (with the parallel production of alcohol) The fermentative production of glycerol may be increased, as it is industrially, by removal of the acetaldehyde, e g., with bisulfite.

In muscle and other tissues, the DPN which is reduced in the course of glycolysis is reoxidized by pyruvic acid itself, which in turn forms lactic acid The $DPNH_2$ can also serve to reduce triose phosphate (as in yeast), with the eventual formation of glycerol required for the synthesis of triglycerides. Dihydroxyacetone phosphate appears to be the triose which is reduced *in vivo*, for it is more likely to yield the L-α-glycerophosphate formed[78] than is D-glyceraldehyde-3-phosphate The free triose is also reduced *to glycerol by the DPN-specific enzyme. The reverse reactions*—phosphorylation of glycerol by ATP and dehydrogenation of the α-glycero-

[74] [illegible] (1950).

[75] [illegible] nd [illegible] 1). [illegible] 0).

[76] [illegible]

[77] E. L Totton and H A Lardy, *J. Biol. Chem* [illegible]

[78] E. Baer and H O L Fischer, *J. Biol Chem* **128**, 491 (1939); **135**, 321 (1940).

phosphate[79]—play a role in the metabolism of glycerol Green has described the properties of a coenzyme-independent dehydrogenase for this reaction [80] Unlike the DPN enzyme, his is associated with the insoluble particles of the cell, and it reduces cytochrome directly (Cf Scheme 2)

c. Oxidation of Triose Phosphate. D-Glyceraldehyde-3-phosphate is the fermentable form of the triose phosphates The first step in its fermentation is a dehydrogenation, the mechanism of which was explained by Warburg and Christian [81] They found that the reaction was catalyzed by inorganic phosphate and unaffected by D-3-phosphoglyceric acid, a putative product. They considered that inorganic phosphate entered into (possibly non-enzymatic) acetal formation with the triose phosphate to form D-1,3-diphosphoglyceraldehyde, which was then oxidized in the presence of DPN and a specific dehydrogenase to the corresponding diphosphorylated acid (with concomitant formation of reduced DPN) The labile "high-energy" 1-phosphate group could then be transferred to ADP yielding D-3-phosphoglyceric acid and ATP In the presence of arsenate, the 1-arseno-3-phosphoglyceric is formed This ester linkage is even more labile than the 1-phosphate, and inorganic arsenate and 3-phosphoglyceric acid appear by spontaneous hydrolysis [81] Not only does the reaction then become irreversible, but the free energy due to the carboxyl phosphate linkage is merely dissipated as heat, no net ATP formation occurring in the presence of arsenate [82]

Yeast diphosphoglyceraldehyde dehydrogenase has been crystallized by Warburg and Christian[81], the muscle enzyme has been crystallized in Cori's laboratory [83] The latter enzyme contains firmly bound DPN as the prosthetic group [84]

The enzyme reversibly transferring the carboxyl phosphate from the 3-carbon ester to ADP, 3-phosphoglycerate kinase, has been found in several tissues It is responsible for the well-known "coupling" of triose phosphate oxidation with ATP formation

d. Conversion of Phosphoglyceric to Pyruvic Acid. Embden showed in 1933 that 3 phosphoglycerate is decomposed by suitable muscle preparations into pyruvic acid and inorganic phosphate [85] Actually this conversion is mediated by three different enzymes, the first effects an ester isomerization in which the phosphate is shifted to the 2 position (phosphoglycero-

[79] F Schlenk, *in* Sumner and Myrbäck, The Enzymes, Academic Press, New York, 1951, Vol 2, Part 1, Chapter 52
[80] D E Green, *Biochem J* 30, 629 (1936)
[81] O Warburg and W Christian, [illegible]
[82] [illegible]
[83] [illegible]
[84] [illegible] *Biol.* [illegible]
[85] G Embden, H. J. Deuticke, and G. Kraft, *Klin. Wochschr* 12, 213 (1933).

mutase) This reaction is somewhat comparable to that catalyzed by phosphoglucomutase in that both involve an intramolecular transfer of phosphate. Moreover, just as phosphoglucomutase requires a diester as coenzyme, so does the phosphoglyceromutase. The ester in the latter case is 2,3-diphosphoglyceric acid,[86] a compound isolated from erythrocytes many years ago[87] The discovery of the equilibrium between the 3- and 2-esters is due to Meyerhof and Kiessling.[88] While the 3-ester preponderates at equilibrium, in glycolysis and fermentation D-2-phosphoglycerate is removed from the reaction by dehydration to enol phosphopyruvic acid Enolase, the enzyme accomplishing this,[82] has been prepared from muscle and yeast Working with yeast enolase which they had crystallized, Warburg and Christian[89] found it to be a mangesium protein. They consider that its marked sensitivity to fluoride[90] is due to replacement of the magnesium on the enzyme by an inactive magnesium fluorophosphate complex

The appearance of inorganic phosphate (along with pyruvic acid) when D-3-phosphoglyceric acid is added to tissue extracts, as in Embden's experiment, is not now considered to occur under physiological conditions The discovery of pyruvate kinase (also known as phosphoenolpyruvate phosphatase) in 1934[90] showed that the enolic ester is dephosphorylated by ADP with the formation of ATP. This reaction, as well as the reverse, i e, the phosphorylation of pyruvic acid, requires the presence of potassium ions[91]

4 THE METABOLISM OF PYRUVIC ACID (SCHEME 3)

a. Phosphorylation of Pyruvic Acid and the Reversal of Glycolysis. *Until* recently the path from pyruvic to the phosphoglyceric acids and thence to glycogen was obscure Attempts to obtain the direct phosphorylation of pyruvic acid were unsuccessful, until it was shown that the formation of phosphoenolpyruvic required, in addition to substrates and enzyme, ATP and K^+ or NH_4^+[91] With this finding the reversibility of all steps in the Meyerhof-Embden-Parnas scheme from pyruvic acid to glycogen has now been demonstrated

b. Lactate Formation. In normal tissues with adequate oxygen supply pyruvic is completely oxidized But under anaerobic conditions (and in certain tissues even aerobically) lactic acid accumulates The reaction, catalyzed by lactic dehydrogenase is as follows:

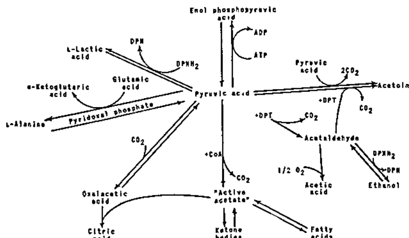
$$\text{Pyruvate} + DPNH_2 \rightleftharpoons \text{L-Lactate} + DPN$$

[86] E W Sutherland, T Posternak, and C F Cori, *J Biol Chem* **181**, 153 (1949)
[87] I Greenwald, *J Biol Chem* **63**, 339 (1925)
[88] [illegible] 220 (1935)
[89]
[90]
[91]

The coenzyme here may be considered to have been reduced in the oxidation of triose phosphate earlier in glycolysis In fact, the pyruvic/lactic and triose phosphate/1,3-diphosphoglyceric reactions appear to be coupled physiologically through DPN (Under aerobic conditions when no lactate is formed, the $DPNH_2$ is reoxidized by diaphorase) Lactic apodehydrogenase has been crystallized from beef heart[92] and from rat skeletal muscle and sarcoma [93]

Unlike the situation in animal tissues, the oxidation of lactic acid to pyruvic in yeast does not require DPN [94]

c. Alcoholic Fermentation. It has been made clear at several points in



SCHEME 3 Reactions of pyruvic acid in animal tissues (See text for details)

the above discussion that glycolysis in animal tissues and alcoholic fermentation are quite comparable This is true throughout the Meyerhof-Embden-Parnas scheme, but the two processes diverge at the pyruvic acid stage In yeast pyruvic acid is decarboxylated to acetaldehyde (through the action of carboxylase) which is then reduced in the presence of $DPNH_2$ to ethanol (alcohol dehydrogenase)

Carboxylase was discovered by Carl Neuberg in 1911 [95] He found that other aliphatic α-keto acids could replace pyruvic Not long before this Harden and Young had prepared a fermenting juice by filtering a yeast slurry under pressure through a gelatin-coated Chamberland filter candle

[92] F B. Straub, *Biochem J* **34**, 483 (1940)
[93] F Kubowitz and P Ott, *Biochem Z* **314**, 94 (1943).
[94] S J Bach, M Dixon, and L G Zerfas, *Biochem J* **40**, 229 (1946).
[95] C Neuberg and L Karczag, *Biochem Z* **36**, 68, 76 (1911).

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$$\text{Pyruvate} + \text{DPNH}_2 \rightleftharpoons \text{L-Lactate} + \text{DPN}$$

[86] E W Sutherland, T Posternak, and C. F Cori, *J Biol. Chem* 181, 153 (1949)

[87] I Greenwald, *J Biol Chem* 63, 339 (1925)

[88] [illegible] 35)

[89] [illegible] 41)

[90] [illegible])

[91] [illegible]

dioxide and an "acetaldehyde-enzyme compound" (possibly an acetaldehyde-DPT compound) This "active" acetaldehyde then reacts with pyruvate to form free enzyme and α-acetolactic acid, the latter subsequently undergoes decarboxylation Added acetaldehyde is not utilized nor is free acetaldehyde produced in the course of acetoin formation by *A aerogenes* [105]

A wheat germ enzyme, α-carboxylase catalyzes acetoin synthesis from either acetaldehyde or pyruvic acid plus acetaldehyde, pyruvic acid alone is a poor substrate.[106]

e. Alanine Formation. L-Alanine is a "non-essential" amino acid for animals; i e , the body can synthesize it How this synthesis occurs was first described by Braunstein and Kritzman [107] Glutamic and pyruvic acids react reversibly in the presence of an "aminopherase," or "transaminase", to give α-ketoglutaric acid and L-alanine. This enzyme has been studied in pigeon breast muscle[107] and in pig heart [108] Pyridoxal phosphate is the coenzyme of the transaminases

f. Oxidative Decarboxylation. In animal tissues the direct decarboxylation of pyruvic acid (forming acetaldehyde, acetoin) is of quite limited importance. On the other hand oxidative decarboxylation is a major pathway for its disappearance In spite of the extensive studies of this pathway in recent years, purification of the enzymes of animal origin has not been accomplished. Greater success, however, has attended the work on the bacterial systems

Acetate itself is formed but slowly in animal tissues and is not a main route for pyruvate metabolism (although acetate administered to animals is completely oxidized) Other products are formed, depending on the enzyme source and other substances present The most important of these products are acetyl phosphate and "active acetate" or acetyl-coenzyme A, the latter being capable of participating in many synthetic reactions involving the condensation of the acetyl group with an acceptor molecule Some of these reactions are the acetylation of foreign amines, the formation of N-acetylglucosamine (a compound occurring in mucoids and mucoproteins) and the formation of acetoacetic acid (an intermediate in fatty acid metabolism) The condensation of "active acetate" (derived, for example, from pyruvic acid) with oxaloacetic acid has been termed the "key reaction" of the tricarboxylic acid cycle.[104] Thus, the oxidative decarboxylation of pyruvic acid to form "active acetate" is the initial step in the com-

[105] M Silverman and C H Werkman, *J. Biol Chem* **138**, 35 (1941)
[106] T. P Singer and J Pensky, *Arch Biochem. Biophys* **31**, 457 (1951); *J. Biol* [illegible]

Their "ultrafiltrate" and residue were each incapable of alcoholic fermentation of glucose, but together they were almost as active as the original juice.[95] The dialyzable, thermostable substance in the ultrafiltrate, provisionally termed "the co-ferment or coenzyme of alcoholic fermentation," was later identified as the coenzyme of carboxylase and finally determined to be diphosphothiamine (DPT).

The acetaldehyde resulting from the decarboxylation is reduced by alcohol dehydrogenase and $DPNH_2$. As in the reduction of pyruvic to lactic in animal tissues, the $DPNH_2$ involved here originates in the course of oxidation of triose phosphate. An alcohol dehydrogenase is also found in mammalian tissues and serves in the oxidation of exogenously introduced alcohol through the acetaldehyde and acetate stages.[97] Its biological significance is not at all clear Coupling of the oxidation of alcohol with the reduction of pyruvate has been demonstrated *in vitro* with liver slices[98] and *in vivo* in the dog.[99]

d. Acetoin Formation from Pyruvate. Enzymes catalyzing the formation of ketols (also known as acyloins, >C(OH)—C(=O)<) from pyruvic acid are fairly widespread. Two pathways exist: either 2 molecules of pyruvic react together to form acetoin (acetylmethylcarbinol), or 1 molecule each of pyruvic and an aldehyde yield the corresponding ketol. Gorr[100] first drew attention to acetoin synthesis in minced heart muscle, this also occurs under appropriate conditions in skeletal muscle, liver, and kidney preparations.[101] Addition of acetaldehyde to these increases the yield of acetoin[101, 102] and decreases the formation of citrate Propionaldehyde and pyruvic acid form acetylethylcarbinol, the next higher homolog of acetoin.[103] The "acetaldehyde" reaction requires DPT and either Mg^{++} or Mn^{++}, phosphate has not been implicated Similar systems occur in yeast and bacteria Acetoin formation seems to be rather a minor pathway of carbohydrate metabolism in animals

The enzymatic components of the ketol reactions have not been purified or separated, so that the mechanism still awaits clarification. However, Ochoa[104] has postulated that the first reaction is the formation of carbon

96 [illegible] 257, 263 (1936)
97 [illegible] *cta*
98 L F Leloir and J M Munoz, *Biochem J* 32, 299 (1938)
99 W W Westerfield, E Stotz, and R L Berg, *J Biol. Chem.* 149, 237 (1943)
100 G. Gorr, *Biochem Z.* 254, 12 (1932)
101 [illegible] *Chem* 264, 91 (1940), E Juni, [illegible]
102 [illegible]
103 R. L Berg and W W Westerfield, *J. Biol Chem* 152, 113 (1944)
104 S. Ochoa, *Physiol. Revs* 31, 56 (1951).

gluconic acid, and, in fact, Dickens[113] obtained evidence that this compound as well as a pentose phosphate is formed

SCHEME 4 The monophosphate shunt (Individual reactions which as yet await demonstration are designated by broken arrows)

This series of reactions has been called upon from time to time to explain the origin of
disconcerting
conic acid wo

[113] F Dickens, *Nature* 138, 1057 (1936), *Biochem J* 32, 1615, 1626, 1645 (1938).

bustion of this compound. However, it should not be construed that the demonstration of these reactions with purified enzymes excludes the possibility of other modes of entrance of pyruvic acid into terminal respiration

g. Carbon Dioxide Fixation. For many years carbon dioxide was thought to be merely a waste product in animal metabolism. Then its role in the chemical control of respiration was discovered. More recently it has been found that carbon dioxide may be fed back into metabolic pathways by carboxylation of certain keto acids, including pyruvic acid An illuminating experiment may be performed by injecting a solution of sodium bicarbonate containing radioactive carbon into an animal, the radioactive "label" can soon be located in many organic compounds extracted from the tissues and body fluids

The fixation of carbon dioxide by pyruvic acid, forming oxalacetic acid, occurs with many bacterial and animal enzyme preparations. It was first demonstrated in bacteria by Krampitz and Werkman[109] and in animal tissues (pigeon liver extract) by Evans, Vennesland, and Slotin.[110]

5. The "Monophosphate Shunt" (Scheme 4)

a. Introduction. Besides the Meyerhof-Embden-Parnas scheme of glycolysis, an alternative pathway of glucose breakdown in yeast and animal tissue has been postulated, based upon work by Warburg, Lipmann, Dickens, and others (Section III 2) This "monophosphate shunt" bypasses the fructose diphosphate stage, the glucose-6-phosphate undergoing stepwise oxidative degradation to glyceraldehyde phosphate. Thus, 1 molecule of glucose yields 3 molecules each of carbon dioxide and water, and 1 of glyceraldehyde phosphate. The last is converted then to pyruvic acid

b. Direct Oxidation of Glucose-6-phosphate. Warburg and Christian found many years ago that a solution of laked erythrocytes is capable of oxidizing glucose-6-phosphate directly.[111] The oxidation depended specifically upon the presence of triphosphopyridine nucleotide (TPN), which is the immediate hydrogen acceptor.[111] The first product is 6-phosphoglucono-lactone [111a] The Robison ester dehydrogenase, named Zwischenferment by Warburg and Christian, has been purified from yeast.

Phosphogluconic acid undergoes a further oxidation in yeast and animal tissues by means of a specific dehydrogenase also requiring TPN Lipmann[112] suggested that the product of this reaction is 2-keto-6-phospho-

[109] L O Krampitz and C H Werkman, *Biochem J* 35, 595 (1941)
[110] E A Evans, Jr , B. Vennesland, and L Slotin, *J Biol Chem* 147, 771 (1943)
[illegible]

[112] F. Lipmann, *Nature* [illegible]

ization of these sugars in energy metabolism via triose phosphate mation.

The further metabolism of phosphorylated pentoses to tetrose phosphate, l of the latter to the triose stage, has thus far been little studied, and sufficient evidence has been adduced to verify this pathway Tetroses re never been identified in the animal body

The synthesis of pentose phosphate by condensation of C_2 and C_3 compnds suggests the possiblity of an analogous reaction in the formation of ribitol (-phosphate) moiety in riboflavin (-phosphate) in microorganis and yeast, for example, by the condensation of triose-3-phosphate l a glycol-like 2-carbon compound

6. Non-Phosphorylative Oxidation of Sugars

The existence of animal enzymes capable of oxidizing free sugars has urally led to the suggestion that still a third pathway for sugar metabon exists Harrison[121] obtained a cytochrome-c linked dehydrogenase m mammalian liver which oxidized D-glucose to D-gluconic acid A 'N- or DPN-linked glucose dehydrogenase has been obtained from ox er by Strecker and Korkes [121a] Notatin, an enzyme obtained from *Aspergilus niger*, also oxidizes glucose, it is a flavoprotein [122] Breusch has found eat liver dehydrogenases for D-arabinose, glyceraldehyde, glycolalde-de, D-erythrose, and the D- and L-threoses [123] In addition, Wainio has served the oxidation of D-xylose, D-lyxose, and D-arabinose by liver eparations [124] The function of these mammalian enzymes which can dize carbohydrates of the series containing from 2 to 6 carbons, inclue, has not yet been related to other patterns of carbohydrate metabolism stepwise degradative oxidation of sugars to carbon dioxide and water uld be a particularly "inefficient" process since coupled phosphorylation d ATP formation) would not occur as in the Meyerhof-Embden-Parnas eme, except possibly in the course of oxidation of the hydrogen atoms

7. Origin of Certain Compounds Related to Hexoses

The success which has attended the *in vitro* synthesis of starch- and ycogen-like compounds by enzymes obtained from animal, plant, and

[120a] Formation of Sedoheptulose-7 phosphate has recently been detected as an intermediary step in pentose metabolism

[121] D C Harrison, *Biochem J* **25**, 1016 (1931), *ibid* **26**, 1295 (1932), J R Hawthorne and D C Harrison, *Biochem J* **33**, 1573 (1939)

[121a] *H. J Strecker and S Korkes, J Biol Chem* **196**, *769 (1952)*

[122] D Müller, *Biochem Z* **199**, 136 (1928), D Keilin and E F Hartree, *Biochem J* **42**, 221 (1948)

[123] F. L Breusch, *Enzymologia* **11**, 87 (1943), *Biochem Z* **321**, 354 (1951)

[124] W. W. Wainio, *J Biol Chem* **168**, 569 (1947)

arabinose-5-phosphate, a sugar not native to animal tissues, although capable on administration of forming liver glycogen.[114] A possible solution to this difficulty has come from the work of Horecker Using purified yeast preparations requiring TPN, he has been able to isolate pentose-5-phosphate[115] *with glucose-6-phosphate as substrate* Scott and Cohen[116] obtained similar results from the oxidation of 6-phosphogluconic acid by a bacterial preparation which could also oxidize glucose- and fructose-6-phosphates Horecker[115] has offered the hypothesis that 6-phosphogluconic acid is oxidized, not at the 2 position as previously assumed, but at the 3 position. Decarboxylation of the 3-keto derivative would account for the observed formation of ribulose-5-phosphate (ketopentose ester) and ribose-5-phosphate,[116a] the later appearing by the action of a phosphopentose isomerase analogous to the well-known phosphohexose isomerase If this scheme is operative in pentose formation in animal tissues as well as yeast, then utilization of a carbon-labeled glycogen precursor by the animal ought to yield the same distribution of isotope in the ribose carbons, numbers 1 to 5, as in the glucose carbons, numbers 2 to 6 Bernstein[117] has performed such an experiment in the chick and was not able to verify the hypothesis.

c. Pentose Formation by Condensation of C_2 and C_3 Units. Recently the aldolase reaction has been reexamined not merely as to substrate specificity but from the point of view of pentose formation and breakdown Schlenk and his colleagues have demonstrated an aldolase type of split of pentose phosphate using animal tissues[118] and bacterial preparations[119] The latter also catalyze a condensation between triose phosphate and glycolaldehyde (or its phosphate) yielding pentose (determined by Mejbaum's orcinol test) Racker[120] has obtained with cell-free bacterial extracts a similar condensation of triose phosphate and acetaldehyde with the formation of what appears to be desoxypentose-5-phosphate (determined by Dische's specific method for the desose). There was evidence that the participating triose phosphate is glyceraldehyde-3-phosphate and not the dihydroxyacetone ester These experiments on the reversible breakdown of pentose phosphates into triose phosphate and a 2-carbon molecule bacterial sources has not been paralleled in the study of the biosynthesis indicate (*a*) a possible route of formation of nucleic acid sugars and (*b*)

[114] E W Rice and J H Roe, *J Biol Chem.* **188**, 463 (1951)

[115] B L Horecker and P Z Smyrniotis, *Arch Biochem* **29**, 232 (1950), *J. Biol Chem* **193**, 371 (1951)

[116] D B M Scott and S S Cohen, *J Biol Chem* **188**, 509 (1951)

[116a] B L. Horecker, P Z Smyrniotis, and J E Seegmiller, *J Biol Chem.* **193**, 383 (1951)

[117] *J Am Chem Soc* **73**, 5003 (1951)

[118] . 185 (1949).

[119] **31**, 154 (1951)

[120]

d. Lactose. Lactose is the only disaccharide synthesized by mammals Analysis of the arteriovenous blood glucose difference in the lactating mammary gland indicates that the glucose uptake is greater than that required for energy purposes of the gland, the excess presumably serving for lactose synthesis Formation of milk sugar from glucose has been achieved with slices of lactating guinea pig mammary gland[129, 130] as well as with fortified homogenates incubated under nitrogen [131] Malpress and Morrison have suggested that either glucose-1-phosphate or glucose-6-phosphate may be an intermediate in lactose synthesis [130]

8 Terminal Oxidation of Carbohydrate

a. Introduction. The Meyerhof-Embden-Parnas scheme of phosphorylative glycolysis represents the main pathway of carbohydrate metabolism in animal tissues and results in the formation of 2 moles of lactate per mole of glucose No net oxygen consumption is involved here, although, of course, there is a dehydrogenation of triose phosphate to phosphoglyceric acid mediated through reduction of DPN Morevoer there is no carbon loss, as carbon dioxide, down to the C_3 stage, as there is in the oxidative schemes The problem of terminal oxidation of carbohydrate thus revolves around the fate of lactic acid or, more specifically, its immediate oxidation product, pyruvic acid, which is on the "main line" of glycolysis Modern biochemistry has viewed the problem of pyruvate oxidation as allied to that of the metabolism of a number of organic acids found to serve as substrates for specific enzymes in plant, animal, and bacterial cells The development of our knowledge of terminal respiration has been presented in a stimulating critique by Krebs [132]

In the first two decades of this century Thunberg,[133] Batelli and Stern,[134] and Lipschitz and Gottschalk[135] made extensive investigations of the capacity of animal tissues to oxidize organic substances Many compounds were found to be oxidized, some of them sufficiently rapidly to warrant the suggestion that they actually play a role in tissue intermediary metabolism This role was provided for in several theories of the terminal oxidation of carbohydrates, the only one of which has stood up to the experimental test being that of Krebs As new information has become available, his original theory has undergone some revision (Scheme 5)

b. The Tricarboxylic Acid Cycle. (1) *Citrate Formation* The formation

[129] G A Grant, *Biochem J* **29**, 1905 (1935)
[130] F H Malpress and A B Morrison, *Biochem J* **46**, 307 (1950).
[131] F J Reithel, M G Horowitz, H M Davidson, and G W Kittlinger, *J Biol. Chem* **194**, 839 (1952)

of the other polysaccharides. This field remains virtually untouched. Many of these carbohydrates have been mentioned in Section II.1, viz , cellulose, arabans, xylans, and polygalacturonides in plant tissues. One may add to this list the animal polysaccharides, hyaluronic acid and heparin. These contain among their hydrolytic products D-glucosamine and D-glucuronic acid

a. D-Glucosamine. Becker and Day[125] prepared glucosone containing radioactive carbon in the aldehyde group. After injection of this compound into rats, the radioactive label was found in blood glucosamine and to a smaller extent in liver glycogen. On the other hand, glucose similarly labeled gave little labeling in the glucosamine, but large amounts in glycogen These experiments indicate that glucose, glucosone, and glucosamine are interconvertible The phosphorylation of D-glucosamine by ATP in various tissues[126] may be the first step in the utilization of this compound for synthesis of polymeric molecules containing it.

b. D-Glucuronic Acid. Phenolic compounds and aromatic acids which gain entrance to the body are excreted in the urine, at least partly, in chemical linkage with D-glucuronic acid. The origin of this compound from D-glucose has been postulated, but some of the experimental evidence does not support the hypothesis Williams,[127] after reviewing the subject, concludes that glucuronic acid used for conjugation is not derived from exogenous glucuronic—which is excreted unchanged for the most part—nor directly from glucose, but rather from a simple C_3 compound (or compounds). The aldehydic carbon atom of glucose is retained in the corresponding position in glucuronic acid.[127a]

c. L-Ascorbic Acid. Like most mammals the rat can synthesize ascorbic acid in its tissues, but the mechanism of its formation is still obscure. It has been shown, however, that its carbon chain can be derived from glucose [128] The transformation is quite complex on paper, since natural ascorbic acid belongs to the L series and is generically derived from the hexose, gulose Ascorbic acid is in equilibirum with dehydroascrobic acid in the tissues, the former predominating When the lactone ring of dehydroascorbic acid is hydrolyzed, L-ketogulonic acid is formed; its lacks vitamin C activity L-Ketogulonic acid is one of the metabolic products of ascorbic acid [128a]

125 [illegible]

126 [illegible] chim [illegible] 1952)

127 [illegible] New York, 1948

127a F. Eisenberg, Jr and S Gurin, *J Biol Chem* **195**, 317 (1952)

128 S S Jackel, E H Mosbach, J J Burns, and C G King, *J. Biol Chem* **186**, 569 (1950)

128a C M Damron, M M Monier, and J H Roe, *J Biol Chem* **195**, 599 (1952)

when any one of these is added The rapid interconversion of these three acids made Krebs' choice of condensation product difficult, but the place of citric acid has now been confirmed experimentally [136, 139]

In 1940–1941 experiments in two laboratories apparently demonstrated that citric acid could not be the initial condensation product [140] Acetate labeled in the carboxyl group with C^{14} was used When added to tissue along with oxalacetate, citrate with only one labeled carboxyl group would be formed Since citric acid is a symmetrical compound, only one-half the amount of label would be expected to appear ultimately in the α-ketoglutaric acid formed by further reactions in the tissue On the contrary, all the C^{14} could be located in one carboxyl group of the α ketoglutarate These experiments indicated that the initial condensation product is an unsymmetrical acid and relegated citric acid formation to a side reaction Ogston then advanced the view that, although citric acid itself is symmetrical, when in the form of enzyme-substrate complex, i e , in its reactive form, it possesses asymmetry [141] This hypothesis was verified by Stern and Ochoa's work[136] and also by Potter and Heidelberger,[139] who incubated labeled acetate and oxalacetate with their tissue preparations and found that the citric acid which could be isolated from the incubation mixture was degraded by other preparations to α-ketoglutarate containing the isotope in the position and amount expected on the basis of the Ogston concept

Martius and Lynen[142] claim that aconitase can catalyze the conversion of citric to isocitric acid directly, that is, without passing through *cis*-aconitic acid

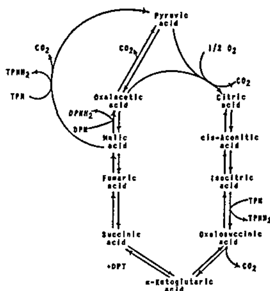
(3) *Formation of α-Ketoglutaric Acid* Isocitric acid is subject to the action of a TPN-linked dehydrogenase, resulting in the formation of oxalosuccinic acid [143, 144] This compound is even less stable than oxalacetic acid, and its decarboxylation to α-ketoglutaric is accelerated by Mn^{++} Another TPN-specific enzyme requiring Mn^{++} for its action decarboxylates isocitrate oxidatively to α-ketoglutarate and carbon dioxide [144] This reaction may be reversed, with fixation of carbon dioxide by mixing α-ketoglutarate and CO_2 with glucose-6-phosphate in the presence of the "isocitric" enzyme and Zwischenferment This is an example of a coupled reaction catalyzed by a trace of TPN

Still another type of isocitric dehydrogenase has been found in yeast [145] It is DPN-specific and also requires AMP It has no action on oxalosuccinic acid. The product of this reaction is α-ketoglutarate

The transamination of α-ketoglutaric acid results in the formation of L-glutamic acid (Section III 4 e)

[139] V R Potter and C Heidelberger, *Nature* 164, 180 (1949)
[140] H G Wood, C H Werkman, A Hemingway, and A O Nier, *J. Biol Chem* 139, 483 (1941), E A Evans, Jr , and L Slotin, *ibid* 141, 439 (1941).
[141] A G Ogston, *Nature* 162, 963 (1948)
[142] C Martius and F Lynen, *Advances in Enzymol* 10, 167 (1950)
[143] S Ochoa, *J Biol Chem* 174, 133 (1948)
[144] A F Graffin and S Ochoa, *Biochim et Biophys Acta* 4, 205 (1950)
[145] A Kornberg and W E Pricer, *J Biol Chem* 189, 123 (1951)

of citrate has recently been achieved with purified enzymes.[136] One system described requires acetate and oxalacetate as substrates; ATP, coenzyme A, and Mg^{++} (or Mn^{++}) as cofactors: and a soluble preparation from acetone-dried pigeon liver as enzyme source. Enzymes in extracts of *E. coli* may be used to convert acetate in the presence of ATP and coenzyme A to "active acetate" The "condensing enzyme" is found in a number of animal tissues, yeast extracts, and bacterial preparations. Fluoroacetate, a highly toxic substance for animals, can also undergo condensation with oxalacetate and the product, fluorocitrate, inhibits the further metabolism



SCHEME 5 The tricarboxylic acid cycle (For details and for other reactions intermediates of the tricarboxylic acid cycle, see text.)

of citrate[137] Accumulation of citrate also results when fluoroacetate injected into animals.[138]

(2) *Aconitase.* Many tissues contain the enzyme aconitase which e tablishes an equilibrium between citric, *cis*-aconitic and isocitric acid

[136] J. R. Stern and S Ochoa, *J Biol Chem* **179**, 491 (1949), **191**, 161 (1951), G I Novelli and F Lipmann, *ibid* **182**, 213 (1950), G G Rudolph and E. S G Barron, *Biochim et Biophys Acta* **5**, 59 (1950); H. Persky and E S G. Barro *ibid* **5**, 66 (1950), V Lorber, M F Utter, H. Rudney, and M. Cook, *J. Bio Chem* **185**, 689 (1950).

[137] W B Elliott and G. Kalnitsky, *J. Biol. Chem* **186**, 487 (1950), P. Buffa, R. Peters, and R. W. Wakelin *Biochem J* **48**, 467 (1951).

[138] P. Buffa and R A Peters, *J. Physiol* **110**, 488 (1949); V. R Potter and H. Busc *Federation Proc* **9**, 215 (1950).

molecule to form malonic acid and CO_2, is catalyzed by myoglobin and Mn^{++}[149]

IV. The Carbohydrate Economy of the Body

1 Homeostatic Processes in Carbohydrate Metabolism

The physiological steady state, exemplified in one instance by the relatively constant blood sugar, is maintained by homeostatic processes involving enzymatic, nervous, and hormonal components The enzymatic aspects of carbohydrate metabolism have already been considered. It should be recognized that the level of sugar in the blood represents a balance between glucose production and glycogenolysis, on the one hand, and glucose utilization, on the other, and consequently gives no indication of the high turnover rate of carbohydrate in the body

The blood sugar is normally maintained at about 0 08% (80 mg per 100 ml) Arterial blood (and capillary blood which it resembles) has a somewhat higher concentration than venous blood in the fed animal but is reduced to the venous level during fasting

Since analytical methods differ in their specificity for sugar, it is important to know how a particular blood sugar concentration was determined before assessing its clinical significance Thus, the method of Folin and Wu analyzes for certain noncarbohydrate reducing substances in blood (which are, however, relatively constant in concentration) and so gives high values (0 09 to 0 12%) Benedict's method is more specific and gives values closer to those for fermentable substances determined as glucose, with a range of 0 07 to 0 10%[150]

2 The Carbohydrate Content of the Body

The chief carbohydrates of the body are glycogen and glucose, but certain other sugars occur in very small amounts The carbohydrate content of the body has been estimated[22] at about 300 g (less than ½ of 1% of body weight in the adult), of which the greater part is glycogen This polysaccharide can be detected in numerous organs, but in widely varying amounts The liver contains a substantial proportion of the body's glycogen store in healthy, well-fed individuals, the actual amount fluctuating considerably with the nutritional state For example, fasting effects a steady reduction in liver glycogen which reaches its minimal values in a few days or less Muscle glycogen is less variable

By administration of C^{14}-labeled glucose and following its changing concentration in the blood stream, it has been possible to estimate experi-

[149] B Vennesland, E A Evans, Jr , and A M Francis, *J Biol Chem* **163**, 573 (1946)

[150] P B Hawk, B L Oser, and W H Summerson, Practical Physiological Chemistry, 12th ed , Blakiston Co , Philadelphia, 1947

(4) *Decarboxylation of α-Ketoglutaric Acid* This occurs primarily as an oxidative reaction i e, with uptake of 1/2 mole of oxygen and formation of 1 mole each of succinic acid and CO_2 Demonstration of succinic acid formation in these enzyme preparations requires the addition of malonic acid (which inhibits the further oxidation of succinic and thus permits it to accumulate) as well as phosphate, Mg^{++}, AMP (or ATP), and DPT. There may be other cofactors not yet recognized. Using certain heart muscle preparations, Green[146] was able to get an anaerobic decarboxylation of α-ketoglutaric acid to succinic semialdehyde.

(5) *The Dicarboxylic Acids* Succinic, fumaric, malic, and oxalacetic figured in the earlier oxidative cycles as well as in a hydrogen transport mechanism once proposed by Szent-Györgyi. Succinic dehydrogenase, along with Green's mammalian α-glycerophosphate dehydrogenase and the yeast lactic dehydrogenase, belongs to the group of dehydrogenases for which the immediate electron acceptor is a member of the cytochrome series The enzyme is also quite stable to treatments which may inactivate other oxidizing enzyme systems of the Krebs cycle.

Fumaric acid undergoes an addition reaction with water to form L-malic acid The enzyme, fumarase, acts only on fumaric, and not on the *cis* isomer, maleic acid, which is toxic to animals Fumarase has recently been crystallized from pig heart muscle by Massey [147]

Two types of enzyme are known to act on L-malic acid. This compound can undergo reversible oxidation to oxalacetic (malic dehydrogenase) or oxidative decarboxylation to pyruvic acid and CO_2 (Ochoa's "malic" enzyme). The first reaction depends upon the presence of DPN. The "malic" enzyme is TPN-specific, requires Mn^{++} (or Mg^{++}) and has an optimum at pH 7 5 [148] At this pH it is inactive toward oxalacetate, but at acid reaction *it can decarboxylate oxalacetate to pyruvate and* CO_2 The oxidative decarboxylation can be reversed by coupling with Zwischenferment or lactic dehydrogenase through TPN [148]

Oxalacetic acid is capable of reacting in a variety of ways: (*a*) reversible reduction to L-malic acid in the presence of reduced DPN and malic dehydrogenase, (*b*) spontaneous decarboxylation, most marked around neutral pH, (*c*) catalyzed decarboxylation, by certain ions and enzymes having a wide distribution, (*d*) condensation with "active" acetate to form citric acid, and (*e*) transamination by glutamic acid to form aspartic acid (*f*) A further reaction, oxidative decarboxylation at the α-carboxyl end of the

[146] D. E Green, W W Westerfeld, B Vennesland, and W E Knox, *J Biol Chem* **145**, 69 (1942)

[147] V Massey, *Biochem J* **51**, 490 (1952)

[148] A H Mehler, A Kornberg, S Grisolia, and S Ochoa, *J Biol Chem* **174**, 961 (1948)

alanine, aspartic acid, and glutamic acid, either through oxidative deamination or transamination, yield keto acids participating in well-known (reversible) routes of carbohydrate breakdown, and can thus contribute to the carbohydrate pool of the body Proline falls into this category also, since an early product in its oxidation is glutamic acid [154] The case of tyrosine is somewhat similar, since it is metabolized through a series of reactions to fumarylacetoacetic acid, which in turn is hydrolyzed to fumaric acid, a Krebs cycle intermediate, and acetoacetic acid [155] Histidine, like proline, is converted to glutamic acid [155a]

b. From Fatty Acids. Conversion of carbohydrates to fat has long been accepted as a normal process in nutrition and is, in fact, of considerable importance in animal husbandry The reverse process, gluconeogenesis from fat, has been the subject of much dispute, but resolution of the problem has finally yielded to experimental trial If the older work was insufficient to establish gluconeogenesis from fat, recent studies have provided a theoretical basis for this conversion Three different routes, all stemming from "active acetate," are available for the metabolic disposal of the fatty acids (Scheme 3). The ultimate products are (*a*) ketone bodies, (*b*) glucose, and (*c*) carbon dioxide and water by combustion of acetate through the Krebs cycle. Ketone bodies are products of an aberrant metabolism, seen in experimental and clinical diabetes, in prolonged fasting as well as in tissue preparations under certain *in vitro* conditions, and they do not normally appear That the transformation does occur has been demonstrated by conversion of butyric acid to carbohydrate in perfusion experiments,[156] by the liver slice technique[157] and by isotope labeling methods [158] The larger question, whether gluconeogenesis from fat occurs to a significant extent in the normal and diabetic animals, has been studied by Strisower *et al* [159] Using C^{14}-labeled palmitic acid, they have estimated conservatively that about 5% of the total glucose turned over in the body glucose pool is from fatty acids in the normal rat, and double this amount in the diabetic rat

c. From Polyhydric Alcohols. The glycogen deposition test reveals that

[154] J V Taggart and R B Krakaur, *J Biol Chem* **177**, 641 (1949)

[155] A B Lerner, *J Biol Chem* **181**, 281 (1949), R G Ravdin and D I Crandall, *ibid* **189**, 137 (1951)

[155a] Y Sera, Osaka Daigaku Igaku Zassi **4**, 1 (1951), [*C A* **46**, 3591 (1952)], A. Abrams and H Borsook, *J Biol Chem* **198**, 205 (1952)

[156] N Blixenkrone-Moller, *Z physiol Chem* **252**, 137 (1938)

[157] M C A Cross and E Holmes, *Brit J Exptl Path* **18**, 370 (1937), H Weil-Malherbe, *Biochem J* **32**, 2276 (1938)

[158] J. M Buchanan, A B Hastings, and F B Nesbett, *J. Biol Chem.* **150**, 413 (1943)

[159] E H Strisower, I L Chaikoff, and E O Weinman, *J. Biol Chem* **192**, 453 (1951)

mentally the size of the body pool of glucose. These amounts are about 0.13% of body weight in the rat[151] and 0.05% in the dog,[152] and include the intracellular as well as the vascular glucose. The method used in establishing these measurements did not yield information on the extent of participation of glycogen in this pool.

3. Gluconeogenesis

The body carbohydrate pool has its origin not merely in dietary carbohydrate but also in the ingested protein and fat. Formation of glycogen (or glucose) from non-carbohydrate precursors is known as gluconeogenesis, a process which has been intensively studied by various techniques. The liver has generally been considered to be the site of gluconeogenesis, but *there is evidence for this function in the kidney also.*[153]

Classical methods utilizing a direct approach to the problem include the use of the diabetic animal, or the normal animal treated with certain drugs. In the former the animal *(usually the dog) is totally depancreatized to induce the diabetic state* ("pancreatic diabetes"). Individual substances can then be fed and the appearance or non-appearance of extra sugar in the urine noted. Administration of *phlorizin* to the normal animal results in glucosuria due to inhibition by this drug of reabsorption of *the glucose from the renal tubules, actually an inhibition of the phosphorylating* mechanism. The other consequences of phlorizin injection resemble those of pancreatic diabetes, except for hypoglycemia. Cessation of the drug results in a return to normal. The phlorizinized animal can be used to study gluconeogenesis in the same way as with the pancreatectomized dog. *Alloxan* has been found to cause, on repeated injection, a necrosis of the insulin-producing tissue of the pancreas, namely the β cells of the islets of Langerhans, and represents a simple alternative to pancreatectomy. Still another technique involves feeding the compound to be tested as a glycogen-*former to animals depleted of their liver glycogen by prolonged fasting,* and then analyzing the liver for its glycogen content after a suitable interval. An increase in liver glycogen content is attributed to synthesis from the administered material.

a. From Protein. By the tests described above the body can utilize protein for carbohydrate formation, but not all the naturally occurring amino acids find their way into the pathways of carbohydrate metabolism. In some cases the deaminated residues form ketone bodies (β-hydroxybutyric acid, acetoacetic acid, and acetone) which are excreted, at least in part, and to that extent make no further contribution to the body's metabolism. It may be said that the classical methods for determining the "gluconeogenic," "ketogenic," or other role of the amino acids have not proved wholly satisfactory and are being supplemented by detailed studies on the metabolic pathways of the individual amino acids. Certain of these, such as

[151] D. D. Feller, E. H. *Strisower,* and I. L. Chaikoff, *J. Biol. Chem.* **187,** 571 (1950).
[152] D. D. Feller, I. L. Chaikoff, E. H. Strisower, and G. L. Searle, *J. Biol. Chem.* **188,**, 865 (1951).
[153] D. R. Drury and E. M. MacKay, *Federation Proc.* **9,** 135 (1950); C. Cohn, B. Katz, and M. Kolinsky, *Am. J. Physiol.* **165,** 423 (1951).

utilization is increased up to a limiting value of about 0 50 g per kilogram per hour attained when the blood sugar approaches 0 50%.[164]

The application of the isotope labeling technique to the problem has permitted such measurements to be made under more nearly normal conditions Feller *et al* have used C^{14}-glucose of high specific activity whereby only a small amount need be injected to obtain readily identifiable labeling in the body glucose and expired carbon dioxide, without significantly altering the ambient blood sugar level Such experiments have been performed, as previously mentioned, in the rat and the dog, and have served to measure, among other things, the rate of glucose oxidation to carbon dioxide and water in these species In the rat, this rate was about 0 7 g per kilogram per hour Alloxanization, resulting in marked hyperglycemia and glycosuria, had relatively little effect on this rate.[151] The normal rate of dissimilation of glucose was lower in the dog, about 0 3 g per kilogram body weight per hour, and slightly less than this in the pancreatectomized animal maintained on an insulin regime [152]

5 Nervous Regulation of Carbohydrate Metabolism

Nervous factors have been shown to play a role, since a variety of brain lesions result in the rapid development of hyperglycemia and glycosuria Claude Bernard first discovered this phenomenon by puncturing the floor of the fourth ventricle (piqûre) and observing the glucosuria which followed Damage to certain areas of the hypothalamus have the same effect, whereas lesions of the paraventricular nuclei cause a hypoglycemia The mechanism of these changes await further elucidation of the tracts and peripheral nerves involved [165]

6 Hormonal Regulation of Carbohydrate Metabolism

a. The Pancreas. (1) *Physiological Functions* An animal whose pancreas is removed develops an elevated blood sugar level which rises above the renal threshold for glucose (about 0 18%), resulting in glucosuria There is an associated polyuria which in turn evokes a polydypsia Ketone bodies appear in the blood and urine, causing acidosis Their presence indicates defective (incomplete) combustion of fatty acids As time goes on, the animal shows signs of wasting (loss of weight, depletion of glycogen stores of the liver first, and later of the muscles, debility) and eventually succumbs in coma The resemblance of this syndrome to that of clinical diabetes in man did not escape the early investigators, who considered a pancreatic lesion or dysfunction to be the cause of diabetes mellitus. The preparation of an extract of the pancreas (insulin) in 1921 which could reverse the

[164] C H Best and N B Taylor, The Physiological Basis of Medical Practice, 4th ed , The Williams and Wilkins Co , Baltimore, 1945

glycerol, sorbitol, and mannitol are gluconeogenic.[160] By a single dehydrogenation step sorbitol yields fructose, the corresponding sugar.[160a] The case of glycerol has already been presented (Section III 3 b).

Inositol, a cyclic hexahydric alcohol, is of interest because of its lipotropic properties It has a widespread occurrence in plants and is also found in small amounts in animals as a constituent of certain cephalins. Fischer's[161] suggestion of its possible relation to glucose (which it resembles configurationally) led to its trial as a gluconeogenic source. When deuterium-labeled inositol was administered to the phlorizinized rat, excess deuterium was found in the urinary glucose.[162] In the fasted, but otherwise normal, rat it did not prove to be glycogenic, although it did prevent the appearance of ketone bodies.[163]

4. The Fate of the Blood Sugar

The peripheral tissues provide a constant drain on the blood sugar The glucose removed from the capillary blood is destined for storage as glycogen, conversion to protein or fat, or breakdown to provide energy. That is, some of the body pool of glucose may be stored temporarily (as glycogen or under limited conditions as lactate) while another portion is removed altogether from the pool through a multitude of reactions, the sum total of which is referred to as utilization of carbohydrate. One can conceive of this "utilized" fraction as being the amount necessary to maintain the body in a steady state with respect to carbohydrate A measure of its size would clearly be a useful index of the rate of carbohydrate metabolism under various conditions Indeed, such estimates have been made, using hepatectomized dogs. In these preparations the blood sugar suffers a steady decline, and the animal undergoes hypoglycemic convulsions unless glucose is infused, in which case death is delayed. The amount of glucose necessary to maintain a constant blood sugar level over an average period of time indicates the rate of utilization Alternatively a detailed chemical balance may be calculated, taking into account changes in the glycogen content of the tissues, the glucose and lactate levels in the blood, and the amount of sugar infused during the experiment; the carbohydrate still unaccounted for is termed the utilized fraction Results by the two methods give rates of the same order (in the dog). about 0 25 g per kilogram per hour[164] By maintaining diabetic blood sugar levels, however, the rate of

[160] C J Carr and J C Krantz, *Advances in Carbohydrate Chem* 1, 175 (1945)

[160a] R. L Blakley, *Biochem J* 49, 257 (1951)

[161] [illegible]

[162] [illegible]

[163] [illegible]

[164] [illegible]

diabetic subject by excessive amounts of sugar was conceived of as being due either to limited capacity of the tissues (especially muscle) to metabolize glucose in the absence of insulin or to an increased rate of hepatic gluconeogenesis, resulting in the production of more glucose than the body can handle Experimental evidence has been adduced for both points of view, but for many years sufficient data of a critical nature were lacking for an unequivocal decision between the "overproduction" and "underutilization" theories

One of the main contentions of partisans of the former theory has been that the tissues of the diabetic animal suffer no inability to utilize glucose The argument is buttressed by the hypoglycemia of the pancreatectomized dog which follows removal of the liver, a phenomenon which would not be expected if the peripheral tissues of the diabetic animal could not metabolize glucose The cogency of the "overproductionist" argument has been weakened, however, by the demonstration (*a*) of a markedly reduced rate of glucose oxidation (admittedly only one of the possible routes of glucose utilization) in uncontrolled pancreatic diabetes in the dog[172] and in alloxan diabetes in the rat,[173] and (*b*) of the increased glucose oxidation in the extrahepatic tissues due to insulinization [174]

Liver slices[175, 176] and diaphragm[177] obtained from diabetic rats also exhibit a decreased glucose oxidation The rate of oxidation of glucose in normal and diabetic liver slices can be increased markedly by pretreatment of the animal with insulin [176] In contrast to the low rate of oxidation of glucose by slices cut from livers of diabetic animals, the oxidation of fructose and acetate is quite normal [178] On the other hand, these substrates are utilized for fatty acid synthesis by diabetic liver slices at a rate less than normal [178] On this basis, Chernick and Chaikoff have postulated that at least two "enzymatic blocks" exist in the diabetic liver (1) at an early stage in glycolysis, and (2) in fatty acid synthesis from a 2-carbon-like intermediate [178] The second block is consistent with the observation of decreased utilization of glucose for the synthesis of fatty acids by the alloxan-diabetic rat[176, 179] and insulin stimulation of fatty acid formation from acetate[180] or glucose [176] in rat liver slices

[173] D Stetten, Jr, I D Welt, D J Ingle, and E H Morley, *J Biol Chem* 192, 817 (1951)

[174] A N Wick, D R Drury, R. W Bancroft, and E M MacKay, *J Biol Chem* 188, 241 (1951)

[175] S S Chernick, I L Chaikoff, E J Masoro, and E Isaeff, *J Biol Chem* 186, 527 (1950)

[176] S S Chernick and I L Chaikoff, *J Biol Chem* 186, 535 (1950)

[177] C A Villee [illegible] (1948)

[178] S [illegible]

[179] D [illegible]

[180] K [illegible]

development of diabetes due to pancreatectomy in animals and also control the condition in man completed the first phase of investigation of the pancreas an an endocrine organ

INSULIN The pancreatic hormone, whose source is in the β cells of the islets of Langerhans, is secreted in response to an elevated blood sugar level The insulin obtained by extraction of the pancreas of one species is active in other species without usually exhibiting antigenic phenomena, in spite of its protein nature. The most pronounced effect of an injection of the purified hormone is a rapid decline in the blood sugar level, which can be readily demonstrated in the normal animal and is, in fact, used in the biological assay of insulin Administration of an excessive amount causes an exaggerated drop in blood sugar, followed by convulsions, which are readily controlled by intravenous administration of glucose or by epinephrine The international unit of insulin is defined as the specific biological activity contained in 1/22 mg. of the crystalline hormone

Insulin can be inactivated by tissue extracts, especially of the liver.[166, 167] The name "insulinase" has been given to the insulin-inactivating system in this organ [167] Its content in rat liver is markedly reduced by fasting but is restored to normal on full feeding [168] Inactivation by blood and excretion in *the urine have also been reported as other fates of insulin in the body.*

THE HYPERGLYCEMIC FACTOR An old observation, namely that injection of insulin sometimes causes a transient rise in the blood sugar preceding the more prolonged fall, led some to propose that such insulins contained an impurity with hyperglycemic activity.[169] This view has been fully vindicated by recent work showing that some commercial, crystalline insulins contain a factor with hyperglycemic and glycogenolytic properties This "H-G" factor may be a product of the α cells of the islets of Langerhans,[170] it can also be extracted from the gastric mucosa.[171] It appears to act by increasing phosphorylase activity in the liver.[172]

(2) *The Role of Insulin* The question which has perhaps proved most intriguing to investigators of carbohydrate metabolism is the nature of the metabolic disturbance in the diabetic state The embarrassment of the

[166] H F Weisberg, A Friedman, and R Levine, *Am J Physiol* 158, 332 (1949)
[illegible]
rt
B
m
10,
825 (1949)
[172] B Zimmerman and T. J Donovan, *Am J. Physiol.* 153, 197 (1948), E W Sutherland and C F Cori, *J Biol Chem* 172, 737 (1948)

subnormal rates.[183] By contrast, when insulin is present in the medium an increased glucose uptake is seen[183, 191] in both normal and "diabetic" diaphragms and in abdominal skeletal muscle,[189] there is also increased glycogen formation.[188, 191-194]

The first step in insulin action seems to be its combination with intact cells,[195] a process which occurs in the first few moments of contact between hormone and tissue. This combining ability is reduced on the part of diaphragm taken from alloxan-diabetic animals.[196]

Krahl and Cori consider that the hexokinase system may not be fully active in the normal diaphragm[183] and that insulin serves to increase its activity and hence glucose utilization. Other work in Cori's laboratory has also associated insulin action with the hexokinase reaction. Thus, it was found that the reaction is inhibited by certain extracts of the anterior pituitary gland *in vivo and in vitro*, and that the inhibition is relieved by insulin, adrenal cortical preparation increased the pituitary inhibition.[197] To view insulin functioning solely as an antagonist to an anterior pituitary–adrenal cortical inhibition omits a positive role in metabolism for this hormone. Moreover it neglects the well-known hypersensitivity of the hypophysectomized animal to insulin. Insulin causes increased glucose utilization by diaphragm taken from hypophysectomized rats,[193] although this theory predicts a lack of influence of insulin on such a process.

Reid, Smith, and Young,[199] while confirming some of the observations made in Cori's laboratory, could find no relation between the *in vitro* potency of anterior pituitary extracts as hexokinase inhibitors and their *in vivo* diabetogenic activity. Broh-Kahn and Mirsky[200] also observed the anterior pituitary-insulin antagonism in occasional experiments, but could

[191] C. A. Villee and A. B. Hastings, *J. Biol. Chem.* **179**, 673 (1949), C. A. Villee, V. K. White and A. B. Hastings, *ibid.* **195**, 287 (1952), E. Walaas and O. Walaas, *ibid.* **195**, 367 (1952)

[192] W. C. Stadie and J. A. Zapp, Jr., *J. Biol. Chem.* **170**, 55 (1947)

[193] O. Riesser, *Biochim. et Biophys. Acta* **1**, 208 (1947)

[194] G. R. Bartlett, A. N. Wick, and E. M. MacKay, *J. Biol. Chem.* **178** 1003 (1949)

[195] W. C. Stadie, N. Haugaard, J. B. Marsh, and A. G. Hills, *Am. J. Med. Sci.* **218**, 265 (1949)

[196] W. C. Stadie, N. Haugaard, A. G. Hills, and J. B. Marsh, *Am. J. Med. Sci.* **218**, 275 (1949)

[197] W. H. Price, C. F. Cori, and S. P. Colowick, *J. Biol. Chem.* **160**, 633 (1945), W. H. Price, M. W. Slein, S. P. Colowick, and G. T. Cori, *Federation Proc.* **5**, 150 (1946), S. P. Colowick, G. T. Cori, and M. W. Slein, *J. Biol. Chem.* **168**, 583 (1947)

[198] J. Bornstein and J. F. Nelson, *Nature* **162**, 572 (1948), M. Perlmutter and R. O. Greep, *J. Biol. Chem.* **174**, 915 (1948), C. H. Li, C. Kalman, and H. M. Evans, *Arch. Biochem.* **22**, 357 (1949), **23**, 512 (1949)

[199] J. Reid, R. H. Smith, and F. G. Young, *Biochem. J.* **42**, xix (1948)

[200] R. H. Broh-Kahn and I. A. Mirsky, *Science* **106**, 148 (1947)

Reference has already been made to the appearance of ketone bodies as a concomitant of the disturbance of carbohydrate metabolism in diabetes and also to the ketogenesis which occurs in liver preparations incubated *in vitro* In the latter case, carbohydrates and carbohydrate intermediates decrease the accumulation of the ketones.[181]

The development of our knowledge of intermediary metabolism and the enzymes concerned therewith has prompted an increasing interest in the last few years in the mechanism of hormone action at the cellular level. Chief among the hormones thus studied has been insulin This *in vitro* work has attempted not only to reproduce in isolated tissues and extracts the metabolic actions of insulin but also to identify which of the numerous enzymatic processes in the cell the hormone specifically affects

A technique introduced by Gemmill in 1940–1941 employing the rat diaphragm muscle as test object[182] has been widely adopted Gemmill observed that when pieces of diaphragm are incubated in a glucose-saline mixture there is uptake of glucose from the medium with net formation of glycogen Glycogenesis is greater in the presence of higher concentrations of glucose than those near physiological levels.[182, 183] Glucose utilization is greater when diaphragms are taken from rats previously fed large amounts of carbohydrate, as against a high fat diet [184] Glycogen formation from glucose has also been shown with liver slices[185, 186, 187] and the internal oblique abdominal muscle[188] of the rat. According to Tuerkischer and Wertheimer[189] the presence of K^+, even in physiological concentrations, inhibits glycogenesis by rat diaphragm, but a high K^+, sodium-free medium is necessary for this process in liver slices [190]

Diaphragm of rats made diabetic by alloxanization takes up glucose at

[181] W C Stadie, J A Zapp, Jr , and F D W. Lukens, *J Biol Chem* **132**, 423 (1940), B G Bobbit and H J Deuel, Jr , *ibid* **143**, 1 (1942), S Weinhouse, R. H Millington, and B Friedman, *J Biol Chem* **181**, 489 (1949), C. H. Beatty and E S West, *ibid* **190**, 603 (1951)

[182] C L Gemmill, *Bull Johns Hopkins Hosp* **66**, 232 (1940), **68**, 329 (1941), C L Gemmill and L Hamman, Jr , *ibid* **68**, 50 (1941)

[183] M E Krahl and C F Cori, *J Biol Chem* **170**, 607 (1947), S S Chernick, I L Chaikoff, and S Abraham, *J Biol Chem* **193**, 793 (1951)

[184] R G Hansen, W J Rutter, and L T Samuels, *J. Biol Chem* **192**, 243 (1951)

[185] O Ostern, D Herbert, and E Holmes, *Biochem J* **33**, 1858 (1939)

[186] C Y. Chiu and D M Needham, *Nature* **164**, 790 (1949), *Biochem J* **46**, 114 (1950)

[187] [illegible]

[188] [illegible] (1949)

[189] [illegible] as been [illegible] and M [illegible]

[190] J M. Buchanan, A [illegible] (1942).

agents, for epinephrine and the hyperglycemic-glycogenolytic factor of the pancreas can increase the amount of active phosphorylase *in vivo* and *in vitro* [207]

c. The Adrenal Cortex. Unlike the medulla this gland is essential for the maintenance of life The adrenalectomized animal suffers from a precarious balance of its carbohydrate metabolism, exhibiting a tendency to hypoglycemia and depletion of the liver glycogen, both of which features become evident when food is withheld Muscle glycogen is more stable than liver glycogen Urine volume decreases, and Na^+ and Cl^- excretion increase These and other sequelae of adrenalectomy may be circumvented when the animal is adequately fed and housed and supplied with isotonic sodium chloride solution, instead of water, to drink In this case the metabolism appears to be normal and remains so unless the animal is subjected to some kind of stressful situation.

A further characteristic of the adrenal-decorticated animal is its hypersensitivity to insulin A physiological antagonism between adrenal cortical hormones and insulin is also seen in the amelioration of pancreatic or alloxan diabetes following adrenalectomy, on the one hand, and the exacerbation of diabetes in animals lacking the pancreas or both pancreas and adrenals by administration of cortical extracts, on the other

Twenty-eight crystalline steroids have been isolated from the adrenal cortex, some of which possess biological activity In essence, those C_{21}-corticoids lacking an oxygen atom at the number 11 position do not affect organic metabolism, although desoxycorticosterone (DOC), the best-known member of this group, can serve to maintain normal salt and water relationships in the adrenalectomized animal and to improve the characteristic circulatory insufficiency The "11-oxygenated" cortical steroids, including corticosterone, 11-dehydrocorticosterone, 11-dehydro-17-hydroxycorticosterone (cortisone, Kendall's compound E) and 17-hydroxycorticosterone (dihydrocortisone, Kendall's compound F), can maintain the life of adrenalectomized animals, and they exert profound effects on the metabolism of carbohydrate and protein On injection into normal animals, for example, these steroids cause glycogen deposition in the liver, and in larger doses [illegible] nce [illegible] tex, [illegible] 17-hydroxycorticosterone is the hormone ordinarily secreted by the human adrenal cortex.[208]

The production of adrenal cortical steroids is regulated by the anterior

[208] P Fourman, F C Bartter, F. Albright, E Dempsey, E Carroll, and J. Alexander, *J Clin Invest* 29, 1462 (1950), J W Conn, L. H. Louis, and S S Fajans, *Science* 113, 713 (1951), K Savard, W J Kolff, and A C Corcoran, *Endocrinology* 50, 366 (1952)

not obtain an effect with diabetic rat tissues. Abood and Gerard[201] have found a fraction in nerve homogenates which inhibits brain, but not yeast or liver, hexokinase. Like the anterior pituitary inhibitor this one is also antagonized by insulin *in vitro* Both are highly labile. A number of groups have not been able to confirm the observations reported from Cori's laboratory.[202]

b. The Adrenal Medulla. Release of epinephrine into the blood stream, either by stimulation of the nerves to the adrenal gland or by administration of the hormone parenterally, results in an increase in blood sugar. This response is also initiated by hypoglycemia such as follows the injection of insulin The underlying sequence of events brought about by epinephrine and leading to the hyperglycemia includes a decrease in the glycogen content of the liver, the immediate source of the extra glucose entering the blood, and glycolysis of muscle glycogen, the muscle lactic acid is swept away by the blood stream to the liver where glucose (or glycogen) is resynthesized. Thus, epinephrine administration may even result in a net increase in liver glycogen. The hyperglycemic action of arterenol (norepinephrine), the second hormone of the adrenal medulla, is much less pronounced [203]

The ability of epinephrine to cause a release of adrenocorticotrophic hormone from the anterior pituitary[204] indicates a link between the sympatho-adrenal system, functioning in what Cannon referred to as "emergencies," and the anterior pituitary-adrenal cortex axis, which is called into play in adaptation to stress [205]

Like insulin, epinephrine binds readily to rat diaphragm [206] It inhibits glycogen synthesis in this tissue[189] and prevents the action of insulin there [193] Thus epinephrine hyperglycemia in the intact animal may be due not only to hepatic glycogenolysis but also to inability of muscle to utilize blood glucose for glycogen synthesis [193] Interestingly enough, this *in vitro* action of epinephrine is abolished by the adrenolytic drug, ergotamine.[189] Active and inactive forms of phosphorylase have been demonstrated in liver and muscle [207] The balance between them seems to be affected by glycogenolytic

201 L G Abood and R W Gerard, *Proc Soc Exptl Biol Med* 77, 438 (1951)

202 W C Stadie and N Haugaard, *J Biol Chem* 177, 311 (1949), W R. Christensen, C H Plimpton, and E G Ball, *ibid* 180, 791 (1949)

203 P Holtz and H J Schumann, *Naturwissenschaften* 35, 159 (1948), E. W McChesney, J P McAuliff, and H Blumberg, *Proc Soc Exptl Biol Med.* 71, [illegible] *J Am Pharm* [illegible]

204 [illegible] ng, *Proc Soc* [illegible] m *J. Physiol* 160, 490 (1950)

205 G Sayers, *Physiol Revs* 30, 241 (1950)

206 W C. Stadie, N Haugaard, and J B Marsh, *J. Biol Chem* 188, 173 (1951)

207 E W. Sutherland and C F Cori, *J Biol Chem* 188, 531 (1951)

tions support the view that some biologically active steroids may exert specific actions at one or more sites in the enzyme systems catalyzing these processes

d. The Anterior Pituitary Gland. Some indication has already been given of the importance of the anterior pituitary gland in carbohydrate metabolism The hypophysectomized animal is characterized by a low blood sugar, which on fasting becomes more marked, even to the point of hypoglycemic convulsions There is also an increased rate of utilization of glucose and decreased glycogen storage Hypophysectomy, like adrenalectomy, ameliorates preexisting pancreatic diabetes and, in fact, the double operation permits the animal to maintain life without insulin Furthermore, it has been demonstrated that injection of diabetogenic extracts of the anterior pituitary can induce a permanent diabetes-like state in the intact animal.

The hypophysectomized animal is particularly susceptible to the action of insulin On the other hand there is an increased insulin resistance by animals injected with anterior pituitary extracts, and this is reflected in the reduced effect of insulin on glycogen formation by diaphragm muscle of rats previously treated with such extracts [214] Normal diaphragms previously incubated with these anterior pituitary preparations also exhibit resistance to the glycogenic effect of insulin *in vitro* [198] Likewise the effect of insulin on diaphragms excised from hypophysectomized rats is substantially increased, in agreement with the *in vivo* hypersensitivity of the hypophysectomized animal to insulin. Stadie *et al* think that this hormonal antagonism may consist of competition between insulin and the pituitary factor for binding sites on the muscle tissue [215] These experiments also show that insulin has *per se* activity at the cellular level and its action is not solely dependent upon the presence of a pituitary factor inhibiting the hexokinase reaction (cf Section IV 6 a)

[214] J. F Nelson, *Australian J Exptl Biol Med Sci* **22**, 131 (1944)

[215] W C Stadie, N Haugaard, and J B Marsh, *J Biol Chem* **188**, 167 (1951)

pituitary through its *adrenocorticotrophic hormone (corticotropin, ACTH)*, the physiological effects of which are essentially the same as those of the 11-oxygenated corticoid group The presence of excessive amounts of the latter compounds in the body (through secretion or by experimental administration) results in a reduced output of corticotropin, so that the anterior pituitary-adrenal cortex axis may be said to possess a somewhat self-regulatory mechanism It should be added that stress evokes an increased output of cortical hormones and that the adrenalectomized animal can respond normally to such environmental pressures only if supplied with exogenous adrenal hormone

Some attempts have been made to elucidate the role of the adrenal cortex and its hormones in intermediary metabolism by *in vitro* studies Villee and Hastings[191] have shown that diaphragm from adrenalectomized rats converts glucose to glycogen at a reduced rate, compared to normals, but the rate can be increased by insulin. There is also in such preparations from adrenalectomized animals a diversion of extra glucose to oxidative and other pathways, indicating the possibility of an inhibitory action of adrenal hormones somewhere below the glucose-6-phosphate level [191]

The glycogenic properties of adrenal cortical hormones are evident not only in the intact animal but also in the isolated, perfused liver[209] and in surviving liver slices.[186, 187] DOC, 11-dehydrocorticosterone, and cortisone *decrease glycogenolysis in rat liver slices and increase glycogen formation*, as well as "total carbohydrate," in the presence of substrate.[187] Results with rat diaphragm are quite different from those with liver, since desoxycorticosterone apparently inhibits glycogen deposition (or stimulates glycogenolysis) [194, 210] The glycogenolytic action of the 11-oxycorticoids is smaller than than of desoxycorticosterone.[211] The relation of these observations to the known effects of the adrenal steroids and of adrenalectomy *in vivo* is not clear, since muscle glycogen is relatively refractory to these treatments, as compared to liver glycogen The presence in the media used in these experiments of potassium ions (which have been reported to inhibit glycogenolysis in diaphragm[189]) may also have contributed to the effect. Riesser[193] found no *in vitro* action using these hormones

Crystalline adrenal corticoids also affect the respiratory metabolism studied *in vitro* For example, DOC inhibits the respiration of slices, as well as homogenates, of various tissues;[212, 213] cortisone has similar, but less, inhibitory activity [213] The effects of these two steroids on the rate of oxidation of certain Krebs' cycle intermediates by adrenal, kidney, and heart prepara-

[209] F L Corey and S W Britton, *Am J Physiol* **131**, 783 (1941)
[210] F Verzar and V Wenner, *Biochem J* **42**, 35 (1948)
[211] F Verzar and V Wenner *Biochem J* **42**, 48 (1948)
[212] M Hayano, S Schiller, and R I Dorman, *Endocrinology* **46**, 387 (1950)
[213] T. L Sourkes and P Heneage, *Endocrinology* **50**, 73 (1952)

I. Introduction

Knowledge of the role of amino acids in nutrition has developed to a large extent from the isolation and characterization of the amino acid components of proteins, and the accumulation of information concerning amino acid metabolism. On the other hand, nutritional studies have frequently indicated the existence of certain metabolic pathways, e g , the conversion of tryptophan to nicotinic acid, and in two cases (methionine and threonine) have resulted in the discovery and characterization of new amino acids The subject of this chapter will be considered in four sections· (1) the discovery and characterization of the natural amino acids, (2) amino acid requirements, (3) general aspects of amino acid metabolism, and (4) the metabolism of individual amino acids

Research in amino acid nutrition and metabolism depends upon a knowledge of the state of existence and chemical properties of the amino acids found in nature, and, from a practical point of view, upon the availability of suitable amounts of these amino acids in pure condition Notable advances in this field are the application of chromatographic techniques to the problems of identification and isolation, and the newer enzymatic methods of amino acid resolution

The dietary requirements of amino acids by various organisms have received considerable attention, although, until relatively recently, such studies were limited to the rat The characterization of an amino acid as "essential" for a certain species and under specific experimental conditions in no way implies that the "dispensable" amino acids are unimportant in nutrition or metabolism It is known that the "dispensable" category of amino acids constitutes a very large proportion of tissue proteins. For example, glutamic acid, "dispensable" in the diet of man and other animals, is one of the most metabolically active amino acids. Its versatility in intermediary metabolism is comparable to that of pyruvic acid. Glutamic acid is an important link between carbohydrate and protein metabolism, and probably represents the most significant pathway for the conversion of ammonia to α-amino groups in higher animals. As a result of the newer

CHAPTER 5

Amino Acids

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1850, it was not isolated from protein hydrolyzates until 1888, when Weyl[5] identified optically inactive alanine as a product of hydrolysis of silk fibroin. Later, Fischer and Skita[6] demonstrated L-alanine in silk fibroin and unequivocally proved the structure L-Alanine was shown to be widely distributed in proteins

(2) L-*Arginine* This amino acid was isolated from etiolated lupine seedlings in 1886,[7] and from a protein hydrolyzate in 1895[8] Later research established the structure[9] Arginine is an important metabolite in the urea cycle in mammalian liver (Section V 9)

(3) L-*Asparagine* The isolation of this compound from asparagus juice[1] and enzymatic hydrolyzates of edestin[10] has been referred to above Asparagine is widely distributed in plants and occurs also in animal tissues

(4) L-*Aspartic Acid* Ritthausen[11] first isolated aspartic acid from protein hydrolyzates, although it was previously known as a product of asparagine degradation It is widely distributed in proteins, in many cases probably as the β-amide It also occurs in the free state in plant and animal tissues.

(5) L-*Cystine and* L-*Cysteine* In 1810, Wollaston[12] isolated cystine from a urinary calculus Many years later, it was demonstrated in horn hydrolyzate by Morner[13] Cystine is pesent in relatively large amounts in keratins Its presence in some hydrolyzates may be attributed in part to oxidation of cysteine Cysteine, the reduced form of cystine, probably also occurs in proteins, as shown by the nitroprusside test for free sulfhydryl groups in certain native proteins

(6) *3,5-Diiodo*-L-*tyrosine* This compound occurs in certain marine animals[14] and in thyroid protein It is probably a biological precursor of thyroxin

(7) L-*Glutamic Acid* This amino acid was isolated from wheat gluten hydrolyzates in 1866 by Ritthausen[15] It is widely distributed in proteins, occurring also as its γ-amide, glutamine L-Glutamic acid is a component of glutathione and folic acid It also occurs in the free state and is very metabolically active, being the chief amino acid involved in transamination. (Section IV 5)

(8) L-*Glutamine* The γ-amide of L-glutamic acid was isolated from beets

[5] T Weyl, *Ber* **21**, 1407 (1888)
[6] E Fischer and A Skita, *Z physiol Chem* **33**, 177 (1901)
[7] E Schulze and E Steiger, *Ber* **19**, 1177 (1886)
[8] S G Bedin, *Z physiol Chem* **20**, 186 (1895)
[9] E Schulze and E Winterstein, *Ber* **30**, 2879 (1897)
[10] M Damodoran, *Biochem J* **26**, 235 (1932)
[11] H Ritthausen, *J prakt chem* (1) **103**, 233 (1868)
[12] W H Wollaston, *Ann chim.* **76**, 21 (1810)
[13] K A H Mörner, *Z physiol Chem* **28**, 595 (1899).
[14] E Drechsel, *Z Biol* **33**, 96 (1896)
[15] H Ritthausen, *J prakt chem* (1) **99**, 454 (1866)

studies on transamination, it is apparent that glutamic acid and its γ-amide, glutamine, play a key role in the exchange of the α-amino groups of most of the amino acids. Interconversions of glutamate and other amino acids, including proline, histidine, ornithine, arginine, citrulline, and γ-aminobutyric acid, as well as the occurrence of glutamate in such molecules as folic acid and glutathione, represent further functions of this amino acid, which clearly cannot be considered "dispensable" in metabolism.

The first step in the catabolism of most amino acids is usually (although not always) deamination Subsequently most of the nitrogen of the α-amino group appears in urea, uric acid, or allantoin. The degradation of the carbon chain may be considered to occur in two phases (1) conversion to compounds which also arise in the course of carbohydrate or fat metabolism, and (2) oxidation of these products to water and carbon dioxide. The first of these phases will be discussed in this chapter.

II. Discovery and Characterization of the Amino Acid

1 Natural Amino Acids

a. Amino Acids Commonly Found in Protein Hydrolyzates. The isolation of asparagine from asparagus juice in 1806 by Vauquelin and Robiquet[1] probably represents the first observation of the presence of an amino acid in natural material Fourteen years later, Braconnot[2] isolated glycine from an acid hydrolyzate of gelatin Thus glycine was the first amino acid to be isolated in pure state from an acid hydrolyzate of protein. Leucine, tyrosine, serine, glutamic acid, aspartic acid, phenylalanine, alanine, lysine, arginine, 3,5-diiodotyrosine, histidine, and cystine were observed as products of protein hydrolysis before 1900, and valine, proline, tryptophan, hydroxyproline, isoleucine, thyroxine, and methionine were recognized as components of protein by 1922 Asparagine and glutamine, the ω-amides of aspartic and glutamic acids, respectively, were established as constituents of protein in 1932, and threonine was isolated in 1936

The amino acids listed below include all but one (hydroxyglutamic acid) of the twenty-one of the "accepted" list of Vickery and Schmidt[3] The criteria established by these authors for "acceptance" were (1) the amino acid "must also have been isolated by some worker other than its discoverer," and (2) "its constitution must have been established by synthesis and by demonstration of identity between the synthetic product and the racemized natural product, or by actual resolution of the synthetic product and preparation of the optically active natural isomer."

(1) L-*Alanine* Although DL-alanine was first synthesized by Strecker[4] in

[1] L N. Vauquelin and P J Robiquet, *Ann chim* 57, 88 (1806).
[2] H Braconnot, *Ann chim et phys* (2), 13, 113 (1820)
[3] H. B Vickery and C L. A Schmidt, *Chem Revs* 9, 169 (1931).
[4] A Strecker, *Ann.* 75, 27 (1850)

(14) L-*Leucine*. Leucine was first obtained from protein in 1819 in an impure state by Proust[31] and shortly thereafter in pure form by Braconnot.[32] The elucidation of structure and synthesis were accomplished in 1891 by Schulze and Likiernik.[33]

(15) L-*Lysine* Drechsel[34] isolated lysine from a casein hydrolyzate in 1889. Its structure was established in 1902 by Fischer and Weigert,[35] and the first crystalline free lysine was obtained in 1928 by Vickery and Leavenworth.[36]

(16) L-*Methionine* This amino acid was discovered in 1922 by Mueller,[37] who noted that it was required for the growth of hemolytic streptococci It is interesting that Osborne[38] noted that the sulfur of proteins was of two types—alkali-labile and alkali-stable—the former representing cystine and cysteine, and the latter as we now know, methionine Barger and Coyne established the structure.[39] The resolution of this amino acid by Windus and Marvel[40] provided further evidence for the proposed structure A toxic derivative of this amino acid, methionine sulfoximine, is formed by the action of agene (NCl_3) on flour.[40a]

(17) L-*Phenylalanine* Schulze and Barbieri[41] discovered this amino acid in etiolated sprouts of *Lupinus luteus* in 1879 Later they demonstrated that phenylalanine was a protein constituent and established the structural identity of the natural and synthetic products

(18) L-*Proline* Proline was synthesized by Willstätter[42] in 1900, and one year later Fischer[43] reported its isolation from casein and the proof of structure

(19) L-*Serine* This amino acid was first isolated from silk by Cramer[44] in 1865, and its structure was later proved by synthesis by Fischer and Leuchs.[45] Serine occurs in many proteins and may be combined with phosphoric acid in some proteins

[31] M Proust, *Ann chim et phys* (2), **10**, 29 (1819)
[32] H Braconnot, *Ann chim et phys* (2), **13**, 113 (1820)
[33] E Schulze and A Likiernik, *Ber* **24**, 669 (1891)
[34] E Drechsel, *J prakt chem* **39**, 425 (1889)
[35] E Fischer and F Weigert, *Ber* **35**, 3772 (1902)
[36] H B Vickery and C S Leavenworth, *J Biol Chem* **76**, 437 (1928)
[37] J H Mueller, *Proc Soc Exptl Biol Med* **19**, 161 (1922)
[38] T B Osborne, The Vegetable Proteins, Longmans, Green and Co , London, 1919
[39] G Barger and F P Coyne, *Biochem J* **22**, 1417 (1928)
[40] W Windus and C S Marvel, *J Am Chem Soc* **52**, 2575 (1930).
[40a] J G Heathcote and J Pace, *Nature* **166**, 354 (1950)
[41] E. Schulze and J Barbieri, *Ber* **12**, 1924 (1879), *Ber* **14**, 1785 (1881).
[42] R Willstätter, *Ber*. **33**, 1160 (1900)
[43] E Fischer, *Z. physiol Chem* **33**, 151 (1901)
[44] E Cramer, *J. Prakt Chem* **96**, 76 (1865).
[45] E Fischer and H Leuchs, *Ber* **35**, 3787 (1902)

in 1883[16] and much later was shown to be a constituent of protein[17] In addition to its presence in proteins, free glutamine occurs in many tissues.

(9) *Glycine.* This amino acid, the first to be isolated from proteins,[2] occurs in many proteins, in the free state, and is a constituent of glutathione, hippuric acid, and glycocholic acid.

(10) L-*Histidine.* Hedin[18] and Kossel[19] independently isolated histidine from proteins in 1896, and its structure was elucidated by later workers. It is widely distributed and occurs in relatively high concentrations in hemoglobin It is a component of carnosine (β-alanyl-L-histidine) and is a precursor of histamine. Methylhistidine has been detected in urine.[20]

(11) *δ-Hydroxy-*L-*lysine* This amino acid was first reported as a constituent of protein hydrolyzates by Van Slyke and Heller.[21] The elucidation of the structure and the synthesis of the amino acid were recently accomplished.[22, 23, 24, 24a, 24b] There still remains the problem of the configurations of the four possible stereoisomers of this amino acid.

(12) *4-Hydroxy-*L-*proline.* This compound was isolated from acid hydrolyzates of gelatin by Fischer[25] in 1902. The synthesis was accomplished by Leuchs,[26] who later succeeded in obtaining all four stereoisomers.[27] Hydroxyproline is present to a considerable extent in collagen and is absent from most of the other proteins which have been studied.

(13) L-*Isoleucine* Ehrlich isolated isoleucine from beet-sugar molasses residue and from a pancreatic digest of fibrin.[28] He noted that it possessed different properties from those of leucine, although its chemical composition [illegible] and succeeded

four

[16] E. Schulze and E Bosshard, *Landw Vers Sta* **29**, 295 (1883)

[17] M Damodoran, G Jaaback, and A E Chibnall, *Biochem J* **26**, 1704 (1932).

[18] S G Hedin, *Z physiol Chem* **22**, 191 (1896).

[19] A Kossel, *Z physiol. Chem* **22**, 176 (1896)

[20] C E Dent, *Biochem J.* **43**, 169 (1948)

[21] D D Van Slyke and A Heller, *Proc Natl Acad Sci U S* **7**, 185 (1921)

[22] [illegible] 2466, 2469, 2472 (1950)

[23] [illegible] 1950)

[24] [illegible]

[24a] [illegible]

[24b] [illegible] *Chem Soc* **73**, 1765 (1951)

[25] E Fischer, *Ber* **35**, 2660 (1902)

[26] H Leuchs, *Ber* **38**, 1937 (1905)

[27] H Leuchs and K Bormann, *Ber.* **52**, 2086 (1919).

[28] F Ehrlich, *Ber* **37**, 1809 (1904)

[29] G D Hiatt, Dissertation, University of Illinois, Urbana, Ill , 1936

[30] J. P. Greenstein, L Levintow, C G. Baker, and J White, *J. Biol. Chem.* **188**, 647 (1951).

certain species by the decarboxylation of aspartic acid (see Section IV.4), although no evidence for this was obtained in the rat [61]

(2) *γ-Aminobutyric Acid* Free γ-aminobutyric acid has been found in brain,[62, 63, 64] yeast,[65] and beetroot [66] It is known to be a product of the decarboxylation of glutamic acid (Section IV 4)

(3) L-*Canavanine* This compound occurs free in soybean and jack bean meals [67] Its synthesis and properties have been described [68] This amino acid has been reported to inhibit the growth of microorganisms [69, 70, 71]

(4) L-*Citrulline* Citrulline occurs in the free state in watermelon juice [72] It was also found in enzymatic digests of casein, although it may have arisen as a result of arginine degradation The significance of citrulline in animal metabolism lies in its role as an intermediate in urea synthesis (Section V 9)

(5) *3,4-Dihydroxyphenyl*-L-*alanine* This compound has been found in extracts of certain plants [73] Its absence from protein hydrolyzates is of uncertain significance in view of the fact that it is readily oxidized

(6) *3,5-Dibromotyrosine* The bromo analogue of iodogorgic acid was isolated by Mörner from the skeleton of the coral *Primnoa lepadifera* in 1913 [74]

(7) L-*Djenkolic acid* This amino acid was isolated from alkali-treated djenkol beans, and also from the urine of humans who had ingested the bean [75] The structure has been established [76, 76a]

(8) *4-Allohydroxy*-L-*proline* This amino acid was isolated from phalloidine, the toxic factor in *Amanita phalloides*, by Wieland and Witkop [77]

(9) L-*Kynurenine* Kynurenine, first reported in 1925,[78] was later found

[61] J R Schenck, *J Biol Chem* **149**, 111 (1943)
[62] J Awapara, A J Landua, R Fuerst, and B Seale, *J Biol Chem* **187**, 35 (1950)
[63] E Roberts and S Frankel, *J Biol Chem* **187**, 55 (1950)
[64] S Udenfriend, *J Biol Chem* **187**, 65 (1950)
[65] L J Reed, *J Biol Chem* **183**, 451 (1950)
[66] R G Westall, *Nature* **165**, 717 (1950)
[67] M Kitagawa and S Monobe, *J Biochem (Japan)* **18**, 333 (1933)
[68] J M Gulland and C J O R Morris, *J Chem Soc* **1935**, 763
[69] N H Horowitz and A M Srb, *J Biol Chem* **174**, 371 (1948)
[70] B E Volcani and E E Snell, *J Biol Chem* **174**, 893 (1948)
[71] H J Teas, *J Biol Chem* **190**, 369 (1951)
[72] M Wada, *Biochem Z* **224**, 420 (1930)
[73] W C Evans and H S Raper, *Biochem J* **31**, 2155 (1937)
[74] C T Mörner, *Z physiol Chem* **88**, 138 (1913)
[75] A G van Veen and A J Hyman, *Rec trav chim* **54**, 493 (1935)
[76] V du Vigneaud and W I Patterson, *J Biol Chem* **114**, 533 (1936)
[76a] M D Armstrong and V du Vigneaud, *J Biol Chem* **173**, 749 (1948)
[77] H Wieland and B Witkop, *Ann* **543**, 171 (1940).
[78] Z Matsuoka and S Yoshimatsu, *Z physiol Chem* **143**, 206 (1925).

(20) L-*Threonine*. Rose and his collaborators[46] isolated threonine from an acid hydrolyzate of fibrin in 1935 It is of interest that the discovery of this amino acid resulted from nutritional studies on rats. The structure and the configurations of the α- and β-carbon atoms were established by Rose and his group. The synthesis of the stereoisomers was accomplished by West and Carter.[47] The assignment of the relative configurations of the isomers is based on chemical[48] and enzymatic[49] procedures.

(21) L-*Thyroxine* Kendall[50] in 1915 isolated this compound from thyroid protein In 1927, Harington and Barger[51] established the structure and synthesized it

(22) L-*Tryptophan* This compound was isolated from an enzymatic digest of casein by Hopkins and Cole in 1901,[52] and the structure was established in 1907 by Ellinger and Flamand.[53] N-Methyl-L-tryptophan has been found in the seeds of *Abrus precatorius*, which grows in Formosa.[54]

(23) L-*Tyrosine* This amino acid was discovered in 1846 by Liebig[55] and isolated from proteins in 1849 by Bopp [56] The structure was established by Erlenmeyer and Lipp [57]

(24) L-*Valine* Although this amino acid was discovered in thymus, thyroid, spleen, liver, and pancreas by von Gorup-Besanez[58] in 1856, it was first reported as a constituent of protein by Schutzenberger[59] in 1879 Fischer[60] established its structure.

b. Other Amino Acids Which Occur in Nature. In addition to those amino acids which have become generally accepted as components of proteins, a number of other amino acids have been found in nature. Some of these may occur in proteins.

(1) *β-Alanine* β-Alanine occurs in carnosine, anserine, pantothenic acid, *coenzyme A, and it has been found in the free state. β-Alanine may arise in*

[46] W C Rose, R H. McCoy, C. E. Meyer, H E Carter, M Womack, and E. T. Mertz, *J Biol Chem.* **109**, 77 (1935)
[47] H D West and H E Carter, *J. Biol Chem.* **119**, 109 (1937).
[48] D F Elliott, *J Chem Soc.* **1950**, 62
[49] [illegible] (1950)
[50] [illegible]
[51] [illegible]
[52] [illegible] (1901)
[53] [illegible]
[54] [illegible] 34, 429 (1941); **36**, 349 (1944)
[55] [illegible]
[56] [illegible]
[57] [illegible] (1883)
[58] [illegible]
[59] P. Schützenberger, *Ann chim et phys* (5), **16**, 289 (1879)
[60] E Fischer, *Z physiol Chem* **33**, 151 (1901), *Ber.* **39**, 2320 (1906)

appears that D-valine as well as its enantiomorph also occur in these hydrolyzates, although there is some doubt as to the relative amounts of D- and L-valine in hydrolyzates of this cyclic polypeptide Inversion during hydrolysis, although theoretically possible, appears unlikely D-Phenylalanine occurs in hydrolyzates of gramicidin-S and tyrocidine [94] Penicillamine (β,β-dimethylcysteine), obtained by acid hydrolysis of penicillin, is an amino acid of the D configuration which is related to D-valine.[95] The polypeptide capsules of strains of *Bacillus anthracis*, *Bacillus subtilis*, and certain other microorganisms contain exclusively D-glutamic acid residues [96-99] However, the cellular glutamic acid of *Bacillus subtilis* is of the L configuration [100]

Although unequivocal demonstration of the occurrence of D-amino acids requires the actual isolation and characterization of the amino acid in question, convincing evidence of an indirect nature for the natural occurrence of certain D-amino acids exists For instance, D-alanine has been found by microbiological assay in hydrolyzates of *Lactobacillus arabinosus*, *Streptococcus faecalis*, and two other organisms [101, 102] Whereas small amounts of vitamin B_6 stimulate the growth of the test organisms, precautions were taken to eliminate this as a source of error D-Alanine was found to stimulate the growth of *Lactobacillus casei* and *Streptococcus faecalis* under conditions where the L isomer was considerably less active A bacterial enzyme system capable of racemizing alanine has been described (see Section V 1 c) There is also evidence for the occurrence of D-glutamic acid in hydrolyzates of *Lactobacillus arabinosus*[103] and D-aspartic acid in *Bacillus brevis* [104] The presence of unidentified D-amino acids in bacteria hydrolyzates as determined with D-amino acid oxidase deserves further study [105]

In summary it may be stated that, although certain D-amino acids occur in nature, their presence in proteins remains to be proven The biological significance of the D-amino acids is as yet in doubt, nevertheless their occurrence in antibiotics is of considerable interest The activity of the antibiotics is probably not dependent upon D-amino acid residues. However,

[94] R L M Synge, *Biochem J* 39, 363 (1945)
[95] H T Clarke, The Chemistry of Penicillin, Princeton University Press, Princeton, N J, 1949
[96] G Ivanovics and V Bruckner, *Z Immunitätsforsch* 90, 304 (1937)
[97] V. Bruckner and G Ivanovics, *Z physiol Chem* 247, 281 (1937)
[98] W E Hanby and H N Rydon, *Biochem J* 40, 297 (1946)
[99] M [illegible]
[100] L [illegible]
[101] J. T [illegible]
[102] J T [illegible]
[103] M [illegible] 43 (1947).
[104] A S. Konikova and N. N Dobbert, *Biokhimiya* 13, 115 (1948)
[105] C. M. Stevens, P. E Halpern, and R P. Gigger, *J. Biol Chem.* 190, 705 (1951).

to be a metabolite of tryptophan (Section V.12). The structure was established in 1942 by Butenandt *et al.*[79]

(10) *Octopine.* Octopine occurs in the free state in scallop and octopus muscle The configuration of the arginine residue is L, and that of the second asymmetric center is probably D [80, 81, 82]

(11) L-*Ornithine* No evidence indicating that ornithine occurs in proteins has yet been obtained, although it may occur in hydrolyzates as a result of the breakdown of arginine. Its importance in the Krebs-Henseleit urea cycle is discussed in Section V 9 This amino acid has been isolated from tyrocidin[83] and gramicidin-S [84]

(12) *Taurine.* This amino sulfonic acid is present in bile in combined form and has been reported to occur in the free state.[20, 85]

(13) *Mimosine* (leucaenine, leucaenol, leucenol) This amino acid was isolated from *Leucaena glauca* Bentham[86] and *Mimosa pudica.*[87] The structure has been proved and its total synthesis reported.[88] Mimosine has a toxic effect on non-ruminants.

c. The Occurrence of D-Amino Acids in Nature. The presence of small amounts of D-amino acids in hydrolyzates of proteins of higher organisms may probably be attributed to inversion or racemization during hydrolysis The claim of Kögl *et al* [89] that certain D-amino acids (in particular D-glutamic acid) were present in tumors has not been substantiated in other laboratories [90] Although D-amino acids have not as yet been demonstrated in the tissues of higher animals, several D-amino acids are known to occur in bacteria and fungi, and it may be anticipated that further study will lead to the discovery of the existence of other D-amino acids in natural material

In 1935 Jacobs and Craig[91] isolated D-proline from acid hydrolyzates of ergot alkaloids, and their findings were confirmed later by other investigators [92] D-leucine was isolated from hydrolyzates of gramicidin,[93] and it

79 A. Butenandt, W Weidel, R. Weichert, and W. von Derjugan, *Z physiol Chem* **279**, 27 (1937)

80 S. Akasi, *J. Biochem (Japan)* **25**, 281 (1937)

81 J L Irvine and D W. Wilson, *J Biol. Chem* **127**, 555 (1939)

82 R. M Herbst and E A Swart, *J Org Chem* **11**, 366 (1946)

83

84

85

86

87

88

89

90 (1950)

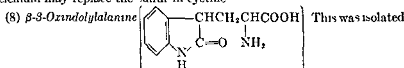
91 W. A Jacobs and C. L. Craig, *J Biol Chem* **110**, 521 (1935)

92 S Smith and G M. Timmis, *J. Chem Soc* **1937**, 396

93 A H Gordon, A J. P. Martin, and R L M Synge, *Biochem J* **37**, 86 (1943)

(6) *Lanthionine* ($(HOOCCHNH_2CH_2)_2S$) The *meso* and DL forms of this compound have been obtained from hydrolyzates of alkali-treated wool and have been synthesized [117-120]

(7) The isolation of a selenium-containing amino acid from *Astragalus pectinatus* grown on a soil rich in selenium has been reported [121] Selenium is incorporated in the proteins of wheat grown on selenium-containing soil. Selenium may replace the sulfur in cystine [122]

(8) *β-3-Oxindolylalanine*


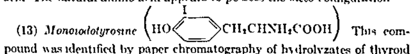
This was isolated from hydrolyzates of phalloidine by Wieland and Witkop [77] This amino acid may not exist in the peptide but may be formed on hydrolysis

(9) L-*Cysteic Acid* ($HO_3SCH_2CHNH_2COOH$) The oxidation of cysteine or hair[123] results in the formation of cysteic acid It has also been reported as a constituent of the outer exposed part of sheep's fleece [124]

(10) *α-Aminobutyric Acid* ($CH_3CH_2CHNH_2COOH$) α-Aminobutyric acid was detected by paper chromatography in normal human urine and the urine of a patient with Franconi syndrome [125]

(11) *α-Amino-β,β-dimethyl-γ-hydroxybutyric acid* ($HOCH_2C(CH_3)_2$-$CHNH_2COOH$) The identification of this amino acid (pantonine) in *E. coli* by paper chromatography has been reported [126]

(12) *α,ε-Diaminopimelic Acid* ($HOOCCHNH_2(CH_2)_3CHNH_2COOH$) This amino acid has been found in *C diphtheriae* It was identified on paper chromatograms and isolated in the crystalline state The properties of the dibenzoyl derivatives of the isolated and synthetic materials were reported to be similar The isolated amino acid was converted to pimelic acid The natural amino acid appears to possess the meso configuration [127]

(13) *Monoiodotyrosine*


This compound was identified by paper chromatography of hydrolyzates of thyroid

[117] M J Horn, D B Jones, and S J Ringel, *J Biol Chem* **138**, 141 (1941), **144**, 87, 93 (1942)

[118] M J Horn and D B Jones, *J Biol Chem* **139**, 473 (1941)

[119] V du Vigneaud and G B Brown, *J Biol Chem* **138**, 473 (1941)

[120] G B Brown and V du Vigneaud, *J Biol Chem* **140**, 767 (1941)

[121] M J Horn and D B Jones, *J Biol Chem* **139**, 649 (1941)

[122] A Fredga, Studien über Selen Di Karbonsäuren und Diselen-Di-Karbonsäuren, Uppsala, Sweden, 1935

[123] T Lissizin, *Z physiol Chem* **173**, 309 (1928)

[124] A J P Martin and R L M Synge, *Advances in Protein Chem* **2**, 1 (1945)

[125] C L Dent, *Biochem J* **40**, xliv (1946), *Science* **105**, 335 (1947)

[126] W W Ackermann and H Kirby, *J Biol Chem* **175**, 483 (1948)

[127] E Work, *Nature* **165**, 74 (1950), *Biochem. J.* **49**, 17 (1951).

the "unnatural" amino acid may render the peptide less susceptible to attack by hydrolytic systems It is of interest in this connection that the cellular glutamate of *B. subtilis* is L, while the capsular glutamate is D

d. Amino Acids Reported To Occur in Nature for Which Complete[3] Proof is Lacking. *(1) α-Aminoadipic Acid* ($HOOCCH_2CH_2CH_2CHNH_2$-$COOH$) Borsook *et al* [106] isolated radioactive α-aminoadipic acid as a degradation product of radioactive L-lysine in liver homogenates. Windsor[107] recently reported the isolation of α-aminoadipic acid as a constituent of a protein in corn steep-water by means of a combined isotopic and chromatographic procedure Elemental analysis of the isolated material was not reported With the same technique, the presence of *free α-aminoadipic acid* in corn seed and corn steep-water and *Aspergillus oryzae* was detected.

(2) *Norleucine* ($CH_3CH_2CH_2CH_2CHNH_2COOH$). The isolation of this amino acid from spinal cord protein was reported in 1901 by Thudichum,[108] and by other investigators in 1913[109] and 1932.[110] Recent studies[111] fail to confirm the earlier reports, however

(3) *Hydroxyglutamic Acid.* Dakin[112] *reported the isolation of this amino* acid in 1918 It was originally accepted as a constituent of protein (ref. 3) However, the occurrence of this amino acid in protein hydrolyzates was questioned by Nicolet and Shinn [113] A repetition of Dakin's isolation by Bailey *et al* [114] led to the conclusion that the isolated product was actually a mixture of other amino acids including glutamic acid and serine.

(4) *α-Aminoheptylic Acid* ($CH_3CH_2CH_2CH_2CH_2CHNH_2COOH$). The natural occurrence of this amino acid was suggested by Polson[115] on the basis of chromatographic studies

(5) *Thiolhistidine* (HC=C—CH_2CHNH_2COOH ring: N, NH, SH). Thiolhistidine occurs in the betaine ergothioneine, obtained from ergot. The possibility of its presence in protein has been considered [116]

[106] H Borsook, C L Deasy, A J Haagen-Smit, G Keighley, and P. H Lowy, [illegible]

333 (1932)

and R. L M Synge,

[112] H D Dakin, *Biochem J* **12**, 290 (1918)

[113] B. H. Nicolet and L A Shinn, *J. Biol Chem* **142**, 139 (1942)

[114] K Bailey, A C. Chibnall, M W Rees, and E. F Williams, *Biochem J.* **37**, 360 (1943).

[115] A Polson, *Biochem et Biophys Acta*, **3**, 205 (1949)

[116] D Blumenthal and H. T. Clarke, *J. Biol Chem.* **110**, 343 (1935).

mediates in biological reactions Some of these are discussed in later sections of this review. This category includes such amino acids as homocysteine, cystathionine, homoserine, and 3-hydroxykynurenine

The possible occurrence of carbamic acid in proteins has been considered [132] It is also conceivable that tricarboxylic amino acids may occur naturally Although oxalosuccinic and isocitric acids have been shown to be important biological intermediates, the analogous-amino acid, α-aminotricarballylic acid has not yet been found in natural material.

Reports have appeared in the literature concerning the isolation of norvaline, hydroxyarginine, hydroxyaspartic acid, α-hydroxyalanine, α-hydroxyvaline, hydroxyleucine, diaminoadipic acid, diaminoglutaric acid, dioxyaminosuberic acid, and "prolysine" [1, 124] Further study is necessary to validate the claims of the occurrence of these amino acids

2 Optical Configuration

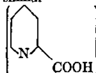
The first observation of the optical activity of amino acids isolated from natural sources was made a century ago by Pasteur [133] Pasteur noted the levorotatory optical activity of natural asparagine and the dextrorotatory activity of aspartic acid and showed that the synthetic aspartic acid of Dessaignes[134] was optically inactive It is surprising that Pasteur considered and discarded the possibility that optically inactive aspartic acid might be an equivalent mixture of dextro- and levorotatory forms It was not until thirty-four years later that experimental evidence was obtained indicating that optically inactive amino acids were actually composed of such mixtures It was found that alkaline hydrolyzates of proteins yielded optically inactive amino acids which frequently exhibited different solubilities than the optically active amino acids obtained by acid hydrolysis of proteins [135, 136] Furthermore, alkaline treatment at high temperatures of optically active amino acids also yielded optically inactive amino acids. Pasteur[137] had reported earlier that *Penicillium glaucum* utilized preferentially the dextrorotatory isomer of racemic tartrate, while its growth left the levorotatory enantiomorph unaffected Utilizing this organism and optically inactive glutamic acid and leucine, Schulze and Bosshard[138] isolated preparations of glutamic acid and leucine which exhibited optical rotations which were opposite in sign and about equal in magnitude to those of the corresponding amino acids isolated from acid hydrolyzates of proteins. These so-called "unnatural" amino acids were the first of the D series to be

[132] A H Corwin and C I Damerel, *J Am Chem Soc* 65, 1974 (1943)
[133] L Pasteur, *Ann* 80, 146 (1851), 82, 324 (1852)
[134] V Dessaignes, *Compt rend* 30, 324, 31, 432 (1850)
[135] E Schulze and E Bosshard, *Z physiol Chem* 10, 134 (1886)
[136] E Schulze, *Z. physiol. Chem.* 9, 63 (1885)
[137] L Pasteur, *Compt rend* 46, 615 (1858), 51, 298 (1860)

tissue.[128] There is evidence that monoiodotyrosine does not represent a breakdown product of diiodotyrosine.[129, 130]

(14) *α,γ-Diaminobutyric Acid* ($CH_2CH_2CHCOOH$ with NH_2, NH_2). The isolation of this amino acid from hydrolyzates of the antibiotic "aerosporin" has been reported [131]

(15) *β-Aminoisobutyric acid* ($H_2NCH_2CH(CH_3)COOH$). This amino acid has been identified in human urine by means of paper chromatography and isolated in crystalline form The properties of the isolated material and a synthetic sample were similar [131a, 131b]



(16) *Pipecolic Acid* [structure] COOH. Preliminary reports describe the isolation of levorotatory pipecolic acid hydrochloride from a number of plant tissues [131c, 131d] This amino acid (a homologue of proline) may arise by cyclization and hydrogenation of the α-keto analogue of lysine.

(17 *β-ε-Diamino-n-caproic acid* ($CH_2NH_2CH_2CH_2CHNH_2CH_2COOH$) This amino acid, a structural isomer of lysine, has been isolated from hydrolyzates of several antibiotics.[131e, 131f] The trivial names "isolysine" and "β-lysine" have been proposed.

(18) *γ-Methylene glutamic acid* ($HOOCCHNH_2CH_2C(=CH_2)COOH$) Evidence for the occurrence of γ-methylene glutamic acid and the corresponding ω-amide in the groundnut, *Arachis hypogaea*, has been reported [131g]

(19) *α-ε-Diamino-β-hydroxypimelic acid* ($HOOCCHNH_2CH_2CH_2CH$-$OHCHNH_2COOH$) This amino acid has been isolated from the phytopathogenic toxin of *Pseudomonas tobaci* [131h]

There are also several amino acids which are believed to occur as inter-

[128] K. Fink and R M Fink, *Science* 108, 358 (1948).
[129] A Taurog, I L Chaikoff, and W Tong, *J. Biol Chem* 178, 997 (1949)
[130] A Taurog, W. Tong, and I. L Chaikoff, *J Biol Chem* 184, 83 (1950)
[131] J R Catch and T S G Jones, *Biochem J* 42, lii (1948).
[131a] H R Crumpler, C E Dent, H Harris, and R. G Westall, *Nature* 167, 307 (1951)
[131b] K. Fink, R B. Henderson, and R M Fink, *Proc Soc Exptl Biol Med* 78, 135 (1951), *J. Biol. Chem* 197, 441 (1952)
[131c] R. M. Zacharius, J F Thompson, and F. C Steward, *J. Am Chem Soc* 74, 2949 (1952).
[131d] A C Hulme and W Arthington, *Nature* 170, 659 (1952)
[131e] H E Carter, W. R. Hearn, E. M Lansford, Jr., A. C. Page, Jr., N. P Salzman D Sharior, and W R. Taylor, *J. Am Chem. Soc* 74, 3704 (1952)
[131f] E E. von Tamelen and E E Swissman, *J. Am Chem Soc.* 74, 3713 (1952)
[131g] J. Done and L. Fowden, *Biochem. J.* 51, 451 (1952)
[131h] D W. Wooley, G Schaffner, and A. C. Braun, *J. Biol. Chem* 198, 709 (1952)

tion, to dextrorotatory malic acid, levorotatory lactic acid, and dextrorotatory tartaric acid The reference standard for the amino acid series is the levorotatory serine commonly found in proteins, which is arbitrarily assigned the L configuration On the basis of the considerations previously discussed, all the amino acids of protein hydrolyzates therefore possess the L configuration Although much indirect evidence indicated that the two standards were in agreement, i e , that the configurations of D-serine and D-glyceraldehyde were similar, direct proof of this relationship was only recently obtained Brewster *et al* [142] have shown that D-glyceraldehyde corresponds in configuration to D-serine by means of a 5-stage genetic route:

$$(+)\text{-glyceraldehyde} \xrightarrow{1} (-)\text{-glyceric acid} \xrightarrow{2}$$
$$(-)\text{-lactic acid} \xrightarrow{3} (+)\text{-}\alpha\text{-bromopropionic}$$
$$\text{acid} \xrightarrow{4} (-)\text{-alanine} \xrightarrow{5} (+)\text{-serine}$$

It had previously been known that (+)-glyceraldehyde could be oxidized to (−)-glyceric acid (stage 1,[143]), and that (+)-glyceric acid could be converted to (+)-lactic acid (stage 2,[144]) The conversion of (+)-α-bromopropionic acid to (+)-lactic acid under conditions controlled to produce inversion was also accomplished (stage 3,[145]) The relationship between (−)-serine and (+)-alanine, established by Fischer,[140] has already been mentioned (stage 5) The final link (stage 4) in the relationship between (+)-glyceraldehyde and (+)-serine was established by converting (+)-α-bromopropionic acid to (+)-alanine by a mechanism involving inversion [142]

The original designations of *d* and *l*, which referred to the direction of rotation in aqueous solution, were abandoned when it became apparent that the amino acids derived from proteins possessed the same configuration. Following a suggestion by Wohl and Freudenberg in 1923, the prefix *l* was used for amino acids of the "natural" series, and the direction of rotation was indicated in parenthesis, e g , *l*(−)-alanine, *l*(−)-cystine, etc However, confusion arose because of the use of water by some workers and acid by others as solvents for optical rotation determinations Furthermore, in the case of threonine, because of its relationship to D-threose, the amino acid isolated from protein was designated *d*(−)-threonine In order to clarify the nomenclature of the amino acids, a set of rules was adopted by British and American chemists [146, 147] Under the new system of nomen-

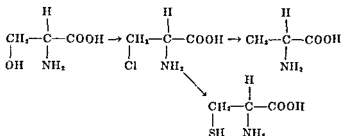
[142] P Brewster, E. D Hughes, C K Ingold, and P A D S. Rao, *Nature* 166, 178 (1950)

[144] A Wohl and R. Schellenberg, *Ber* 55B, 1404 (1922)

[illegible]

prepared Later workers employed the same principle in obtaining other D-amino acids Thus Ehrlich[138, 139] obtained D-alanine, D-valine, and D-isoleucine by the action of growing yeast on the corresponding racemates More recently, purified enzyme preparations have been used to prepare D-amino acids from racemates (see Section II.3).

In the early literature, the amino acids are preceded by the prefix *d* or *l*, indicating the *dextro* or *levo* direction of the optical rotation of an aqueous solution of the amino acid. Thus, for example, *d*-aspartic acid, *l*-histidine, *l*-leucine, and *d*-valine were the designations employed for the isomers obtained from acid hydrolyzates of protein However, it became apparent that all the amino acids derived from proteins possessed the same configuration about the α-carbon atom This conclusion was based in part upon the demonstration that a number of amino acids could be converted into each other or to identical compounds, by reactions not involving the α-carbon atom Thus Fischer[140] converted levorotatory serine to dextrorotatory alanine and levorotatory cysteine:



Evidence based on comparisons of changes of optical rotation due to ionization and substitutions in analogous amino and hydroxy acid derivatives also gives support to this conclusion [141] Information derived from studies on optically specific enzyme systems is also compatible with the concept that the amino acids derived from proteins are of the same configuration Thus D-amino acid oxidase attacks only D-amino acids, and L-amino acid oxidase exhibits a similar stereochemical specificity Certain preparations of the latter enzyme also attack L-α-hydroxy acids, thereby providing evidence relating the configuration of the L-α-hydroxy acids and the L-α-amino acids There are numerous examples of the strict stereospecificity of enzyme systems.

The accepted standard of reference for the configuration of the carbohydrates is the dextrorotatory isomer of glyceraldehyde (D-glyceraldehyde) This compound may be chemically converted, without change of configura-

138
139 (1908)
140 , 893 (1908)
141 1948)

recent reviews[157, 158, 159] The development of chromatographic methods has enormously simplified the isolation of amino acids[159a, 159b]

b. Preparation of One Isomer from a Racemate by Selective Destruction of Its Enantiomorph. The early work of Schulze and Bosshard,[135] who prepared D-glutamic acid and D-leucine from the respective racemates by the action of a mold, has been mentioned Other D-amino acids, i e , D-alanine,[138] D-valine,[160] D-isoleucine,[138] D-histidine,[138] D-phenylalanine,[139] D-tyrosine,[160] and D-serine,[139] have been prepared by the selective action of yeast or other microorganisms on the L isomers Animals fed or injected with certain racemic amino acids have been found to excrete large amounts of the D isomers in the urine This permitted the use of whole animals for the preparation of certain D isomers, e g , D-histidine[161] Destruction of a single isomer of a racemate may also be accomplished with enzyme preparations. In 1906, Riesser[162] prepared D-arginine from DL-arginine using liver arginase The L-amino acid oxidase of *Proteus vulgaris*[163] was used to obtain D-leucine, D-methionine, and D-phenylalanine, and the D-amino acid oxidase of mammalian kidney has been employed for the preparation of L-alanine,[164, 165] L-methionine,[165] and L-proline[166] D-Lysine[167] and D-glutamic acid[168] have been prepared by employing the corresponding optically specific decarboxylases

c. Resolution by Chemical Procedures. The disadvantage in procedures involving selective destruction of one isomer is, of course, that only one enantiomorph of the racemate is obtained A further difficulty with the procedures utilizing growing microorganisms lies in the fact that some destruction of the D isomer usually occurs A method by which both isomers could be obtained from the same racemate (resolution) was first developed by Fischer in 1899 Fischer, applying the Pasteur principle of diastereo-

[157] R. J Block, *Chem Revs* 38, 501 (1946)
[158] H O Calvery in Schmidt, Chemistry of the Amino Acids and Proteins, Charles C Thomas, Springfield, Ill , 1938, p 123
[159] M S Dunn and L B Rockland, in Greenberg, Amino Acids and Proteins, Charles C Thomas, Springfield, Ill , 1951, p 213
[159a] W H Stein and S Moore, *Cold Spring Harbor Symposia Quant Biol* 14, 179 (1950)
[159b] C H W Hirs, S Moore, and W H Stein, *J Biol Chem* 195, 682 (1952).
[160] F Ehrlich, *Z physiol Chem* 181, 140 (1929)
[161] M Koyokawa, *Z physiol Chem* 214, 38 (1933)
[162] O Riesser, *Z physiol Chem* 49, 210 (1906)
[163] P K Stumpf and D E Green, *J Biol Chem* 153, 387 (1944).
[164] O K Behrens, *J Biol Chem* 141, 465 (1941)
[165] R Duschinsky and J Jeanneret, *Compt rend.* 208, 1359 (1939).
[166] M R Stetten and R Schoenheimer, *J Biol Chem* 153, 113 (1944).
[167] A Neuberger and F Sanger, *Biochem J.* 38, 125 (1944)
[168] M M Camien, L E McClure, and M S Dunn, *Arch. Biochem* 28, 220 (1950)

clature, the use of the small capital letters L and D are employed to designate the two series of amino acids. In order to avoid ambiguities resulting from possible confusion with the nomenclature of the carbohydrates, the subscripts *g* (for glyceraldehyde) and *s* (for serine) are employed to specify the system of nomenclature. Thus D_g-mannosaminic acid is also an L-amino acid

The configuration and the direction of the optical rotation in water and in acid of the natural amino acids are given in Table 1.

Several amino acids possess two asymmetric carbon atoms and therefore may exist in four stereoisomeric forms Reference has already been made to the preparation of the stereoisomers of isoleucine, hydroxyproline, and threonine The β-carbon atom of L-threonine and D-allothreonine is of the D configuration[148] The configuration of the γ-carbon atom of hydroxy-L-proline is also D. In hydroxy-L-proline and hydroxy-D-proline, the hydroxyl group is in *trans* position to the carboxyl group, whereas in allohydroxy-L-proline and allohydroxy-D-proline the relationship between the carboxyl and hydroxy groups is *cis*.[149, 150, 151] The configurations of the second asymmetric centers of isoleucine and 5-hydroxylysine are not known.

Racemization in acid of cystine leads to a *meso* form (which cannot be resolved by procedures capable of resolving DL-cystine) as well as some DL-cystine[152, 153] Similar studies of homocystine and djenkolic acid have apparently not been reported The *meso* and optically active forms of lanthionine have been prepared[117-120] In natural octopine the arginine moiety is unquestionably L, while the second center of asymmetry is probably of the D configuration. "Isooctopine," possessing the LL configuration, has also been prepared[154, 155, 156]

3 Preparation of L and D Isomers of Amino Acids

a. Isolation of L Isomers. An extensive literature on the isolation of amino acids from protein hydrolyzates has accumulated Techniques which have been employed for isolating amino acids from hydrolyzates of proteins include isoelectric precipitation, isolation as salts, esters, hydrochlorides, and sulfonic acids, electrodialysis, ionophoresis, and chromatography These procedures have been described in detail and discussed in several

[148] C E Meyer and W C Rose, *J. Biol Chem* **115**, 721 (1936)
[149] A Neuberger, *J Chem Soc* **1945**, 429
[150] T. Kaneko, *J Chem Soc (Japan)* **61**, 207 (1940) [*C A* **37**, 117 (1943)]
[151] C S Hudson and A. Neuberger, *J. Org Chem.* **15**, 24 (1950)
[152] [illegible]
[153] [illegible]
[154] [illegible]
[155] [illegible]
[156] S Akasi, *J. Biochem. (Japan)* **25**, 281 (1937).

TABLE 1—*continued*

Amino acid	Chemical formula	Direction of optical rotation: In water	Direction of optical rotation: In acid
4-Hydroxy-L-proline	HO—CH—CH_2; CH_2 CH—COOH; NH (ring)	−	−
4-Allohydroxy-L-proline	(same formula)	−	
L-Isoleucine	CH_3, C_2H_5 > $CHCHNH_2COOH$	+	+
L-Kynurenine	O ‖ C—CH_2CHNH_2COOH on benzene ring with NH_2	−	
L-Leucine	CH_3, CH_3 > $CHCH_2CHNH_2COOH$	−	+
D-Leucine		+	−
L-Lysine	$CH_2NH_2CH_2CH_2CH_2CHNH_2COOH$	+	+
L-Methionine	$CH_3SCH_2CH_2CHNH_2COOH$	−	+
Mimosine	HO; O= (pyridone ring) N—CH_2CHNH_2COOH	−	+
Octopine	$H_2NCNHCH_2CH_2CH_2CHNHCHCH_3$ (‖ NH; COOH; COOH)	+	
L-Ornithine	$CH_2NH_2CH_2CH_2CHNH_2COOH$	+	+
L-Phenylalanine	(benzene ring)CH_2CHNH_2COOH	−	−
D-Phenylalanine		+	+
L-Proline	CH_2—CH_2; CH_2 CHCOOH; NH (ring)	−	−
D-Proline		+	+
L-Serine	$HOCH_2CHNH_2COOH$	−	+

TABLE 1

NATURAL AMINO ACIDS

Amino acid	Chemical formula	Direction of optical rotation: In water	Direction of optical rotation: In acid
L-Alanine	CH_3CHNH_2COOH	+	+
β-Alanine	$CH_2NH_2CH_2COOH$		
γ-Aminobutyric acid	$CH_2NH_2CH_2CH_2COOH$		
L-Arginine	$H_2NCNHCH_2CH_2CH_2CHNH_2COOH$ ‖ NH	+	+
L-Asparagine	$H_2NOCCH_2CHNH_2COOH$	−	+
L-Aspartic acid	$HOOCCH_2CHNH_2COOH$	+	+
L-Canavanine	$H_2NCNHOCH_2CH_2CHNH_2COOH$ ‖ NH	+	
L-Citrulline	$H_2NCNH—CH_2CH_2CH_2CHNH_2COOH$ ‖ O	+	+
L-Cysteine	$HSCH_2CHNH_2COOH$	−	+
L-Cystine	$[—SCH_2CHNH_2COOH]_2$	−	−
3,5-Dibromo-tyrosine	Br HO⟨benzene ring⟩CH_2CHNH_2COOH Br		
3,4-Dihydroxy-phenyl-L-alanine	HO HO⟨benzene ring⟩CH_2CHNH_2COOH	−	−
3,5-Diiodo L-tyrosine	I HO⟨benzene ring⟩CH_2CHNH_2COOH I		+
L-Djenkolic acid	$HOOCCHCH_2SCH_2SCH_2CHNH_2COOH$ | NH_2		−
L-Glutamic acid	$HOOCCH_2CH_2CHNH_2COOH$	+	+
D Glutamic acid		−	−
L-Glutamine	$H_2NOCCH_2CH_2CHNH_2COOH$	+	
Glycine	CH_2NH_2COOH		
L-Histidine	HC═══C—CH_2CHNH_2COOH | | HN N \ // CH	−	+
5-Hydroxy-L-lysine	$CH_2NH_2CHOHCH_2CH_2CHNH_2COOH$		

benzoyl-D-alanine unaffected. Later, Fruton, Irving, and Bergmann[176] found that papain, in the presence of N-carbobenzoxy-DL-amino acids and aniline, catalyzed the enzymatic synthesis of N-carbobenzoxy-L-amino acid anilides more rapidly than the corresponding D-anilides. Since the insoluble L-anilide precipitated first, a separation of isomers was achieved. This method has been used in resolving racemic phenylalanine,[177] methionine,[178] tryptophan,[179] S-benzylhomocysteine,[180] and other amino acids.[181]

A general enzymatic procedure for the resolution of racemic amino acids has been developed by Greenstein and his collaborators.[49, 182-194c] This procedure makes use of the observation that, when N-acylated racemic amino acids are subjected to attack by an enzyme (acylase) present in hog or rat kidney, only the L isomer is attacked. At the completion of the enzymatic reaction, all the L isomer is present as the free amino acid, while the acylated D-amino acid remains intact. These may be readily separated in a single step since the free L-amino acid is completely insoluble in non-aqueous solvents in which the N-acyl-D-amino acid is completely soluble. In some

[176] J. S. Fruton, G. W. Irving, Jr., and M. Bergmann, *J. Biol. Chem.* **133**, 703 (1940)

[177] O. K. Behrens, D. G. Doherty, and M. Bergmann, *J. Biol. Chem.* **136**, 61 (1940)

[178] C. A. Dekker and J. S. Fruton, *J. Biol. Chem.* **173**, 471 (1948)

[179] H. T. Hanson and E. L. Smith, *J. Biol. Chem.*, **179**, 815 (1949)

[180] L. J. Reed, A. R. Kidwai, and V. du Vigneaud, *J. Biol. Chem.* **180**, 571 (1949)

[181] D. G. Doherty and E. A. Popenoe, Jr., *J. Biol. Chem.* **189**, 447 (1951)

[182] P. J. Foder, V. E. Price, and J. P. Greenstein, *J. Biol. Chem.* **178**, 503 (1949)

[183] V. E. Price, J. B. Gilbert, and J. P. Greenstein, *J. Biol. Chem.* **179**, 1169 (1949)

[184] J. B. Gilbert, V. E. Price, and J. P. Greenstein, *J. Biol. Chem.* **180**, 473 (1949).

[185] J. P. Greenstein, J. B. Gilbert, and P. J. Fodor, *J. Biol. Chem.* **182**, 451 (1950)

[186] L. Levintow, V. E. Price, and J. P. Greenstein, *J. Biol. Chem.* **184**, 55 (1950)

[187] J. P. Greenstein, L. Levintow, C. G. Baker, and J. White, *J. Biol. Chem.* **188**, 647 (1951)

[188] L. Levintow and J. P. Greenstein, *J. Biol. Chem.* **188**, 643 (1951)

[189] L. Levintow, J. P. Greenstein, and R. B. Kingsley, *Arch. Biochem. Biophys.* **31**, 77 (1951)

[190] C. G. Baker and A. Meister, *J. Am. Chem. Soc.* **73**, 1336 (1951)

[191] D. Hamer and J. P. Greenstein, *J. Biol. Chem.* **193**, 81 (1951)

[192] S. M. Birnbaum, L. Levintow, R. B. Kingsley, and J. P. Greenstein, *J. Biol. Chem.* **194**, 455 (1952)

[193] D. Rudman, A. Meister, and J. P. Greenstein, *J. Am. Chem. Soc.* **74**, 551 (1952)

[194] S. M. Birnbaum and J. P. Greenstein, *Arch. Biochem. Biophys.* **39**, 108 (1952)

[194a] S. M. Birnbaum, R. J. Koegel, S.-C. J. Fu, and J. P. Greenstein, *J. Biol. Chem.* **198**, 335 (1952)

[194b] C. G. Baker, S.-C. J. Fu, S. M. Birnbaum, H. A. Sober, and J. P. Greenstein, *J. Am. Soc. Chem.* **74**, 4701 (1952)

[194c] S.-C. J. Fu, K. R. Rao, S. M. Birnbaum, and J. P. Greenstein, *J. Biol. Chem.* **199**, 207 (1952)

TABLE 1—*continued*

Amino acid	Chemical formula	Direction of optical rotation: In water	Direction of optical rotation: In acid
Taurine	$CH_2NH_2CH_2SO_3H$		
L-Threonine	$CH_3CHOHCHNH_2COOH$	−	−
L-Thyroxine	HO–(3,5-diiodophenyl)–O–(3,5-diiodophenyl)–CH_2CHNH_2COOH		−
L-Tryptophan	(indol-3-yl, N–H)–CH_2CHNH_2COOH	−	+
L-Tyrosine	HO–(phenyl)–CH_2CHNH_2COOH	−	−
L-Valine	$(CH_3)_2CHCHNH_2COOH$	+	+
D-Valine	$(CH_3)_2CHCHNH_2COOH$	−	−

isomerism, mixed the N-acylated racemic amino acid with optically active alkaloids, (e g , brucine, strychnine, cinchonine, quinidine, quinine) thus obtaining two diastereoisomeric forms possessing different solubilities These were separated in suitable solvents, the alkaloid was regenerated, and the acyl group hydrolyzed By this general procedure most of the amino acids were resolved by Fischer and his collaborators[169-172] and by later workers [173]

d. Biological Methods of Resolution. In 1905, Warburg[174] reported an asymmetric action of trypsin on DL-leucine ethyl ester, whereby only the ester of the L isomer was hydrolyzed In 1924, Neuberg and Linhardt[175] observed that takadiastase split L-alanine from benzoyl-DL-alanine, leaving

169 E Fischer, *Ber* **32**, *2451*, *3638 (1899)*, ***33***, *2370 (1900)*, ***39***, *530*, *2390*, *2942 (1906)*
170 E Fischer and A Mouneyrat, *Ber* **33**, 2383 (1900)
171 E. Fischer and W Schoeller, *Ann* **357**, 1 (1907)
172 E Fischer, *Ber* **42**, 2989 (1909)
173 [illegible] *Chem* 3, 205 (1947)
174 [illegible]
175 [illegible]

L-isoleucine or a mixture of the four stereoisomers DL-Alanine preparations may contain glycine, L-tyrosine is frequently contaminated with cystine, tyrosine may be present as an impurity in samples of L-tryptophan, and L-glutamine may contain arginine and asparagine The use of amino acids as supplied by a manufacturer, without purification or characterization, is clearly undesirable

In general, to be pure, an amino acid should be white and crystalline and give the correct analytical figures for the elements it contains Detection of contamination with other amino acids can be readily determined by a series of paper chromatograms in a number of different solvents In some cases microbiological assay, solubility measurements, Van Slyke gasometric (nitrous acid and ninhydrin) determinations, titration, and certain special procedures may be useful The procedure for the determination of optical purity by means of optically specific enzymes[200] is capable of detecting at least a 0 1% contamination of one isomer by its enantiomorph. In at least two cases (L-alanine and L-serine) contamination of one isomer by its enantiomorph can be detected when present to the extent of 1 part in 10,000 The determination of specific optical rotation is of some value in establishing the purity of certain amino acids The limitations of this procedure, as a criterion of optical purity, have been discussed [200]

III. Amino Acid Requirements

1 Higher Animals

The need for protein in the diet of higher animals has long been recognized It is also well established that certain proteins are capable of supporting growth whereas other proteins cannot Since proteins are hydrolyzed to the component amino acids within the alimentary canal, the biological value of a protein might logically be expected to depend upon its amino acid composition That those proteins which were nutritionally inadequate were deficient in one or more constituent amino acids followed (a) from experiments in which such proteins were supplemented by certain amino acids, and (b) by total amino acid analyses of the proteins Advances in this field have depended to a large extent on quantitative analysis of the amino acid components of the proteins, and upon superior techniques of isolating and identifying the amino acids. As a result of such necessary supplementation, the concept arose that certain amino acids were synthesized in the body, whereas others, the so-called "essential" amino acids, had to be present in the diet and were presumably not synthesized in the body. With the accumulation of experimental findings, the original concepts of "essential" and "non-essential" must be modified. The definition of an

[200] A. Meister, L. Levintow, R. B Kingsley, and J. P. Greenstein, *J. Biol. Chem* 192, 535 (1951).

cases (e g , histidine, S-benzylcysteine, proline) the amides of the racemic amino acids and a kidney amidase were used. Originally a beef pancreas preparation was used for the resolution of phenylalanine, tryosine, and tryptophan Owing to the development of a purified kidney acylase preparation by Birnbaum *et al.*,[192] these amino acids can be readily resolved with the same preparation used for the other amino acids It is of interest that the hydrolysis of acylated L-aspartic acid is catalyzed by a separate enzyme (acylase II) also present in kidney. Furthermore, the kidney enzyme which hydrolyzes L-proline amide is not identical with the amidase which acts on the amides of S-benzylcysteine or histidine. By means of Greenstein's procedure, the following racemic amino acids were readily resolved and the pure isomers obtained: alanine,[182, 184] methionine,[185] valine,[183] threonine,[183] serine,[183] leucine,[193] glutamic acid,[183, 189] aspartic acid,[183] phenylalanine,[184] tyrosine,[184] tryptophan,[184] lysine,[185] histidine,[186, 194] cystine,[186, 194] isoleucine,[187] ornithine,[188] arginine,[188, 194] and proline.[191] In addition the method can be used to obtain the isomers of allothreonine,[49] alloisoleucine,[187] ethionine,[192] β-cyclohexylalanine,[193] and a number of other unnatural amino acids [185, 190, 193-195]

Independently, Brenner *et al* [196] developed a resolution procedure based on asymmetric enzymatic hydrolysis of amino acid esters. The chief disadvantage of this procedure lies in the fact that some spontaneous hydrolysis of the amino acid esters occur, so that the L form is contaminated with variable amounts of the D isomer. The procedure has been employed for the resolution of tryptophan,[196] methionine,[197, 198] phenylalanine,[199] and valine.[199a]

4. Purity of Amino Acids

All samples of amino acids to be used in biochemical or nutritional work should be checked for purity. This is of importance not only for those amino acids synthesized, resolved, or isolated by the investigator, but also for samples obtained from commercial sources At this writing, many impure amino acids are on the market Preparations of isolated amino acids frequently contain small amounts of other natural amino acids L-Glutamic acid and L-leucine preparations may contain methionine, samples of leucine (L or DL) may contain as much as 20% isoleucine, and "DL-isoleucine" has frequently been found to be an epimeric mixture of D-alloisoleucine and

[194d] L Berlinguet and R Gaudry, *J. Biol Chem* **198**, 765 (1952)

[195] B F Crowe and F F Nord, *J Org Chem* **15**, 688 (1950).

[196] M Brenner, E Sailer, and V Kocher, *Helv Chim Acta* **31**, 1908 (1948)

[197] M Brenner and V Kocher, *Helv Chim Acta* **32**, 333 (1949)

[198] K A. J Wretlind and W C Rose, *J Biol Chem* **187**, 697 (1950).

[199] K A J Wretlind, *J Biol Chem* **186**, 221 (1950)

[199a] K. A J Wretlind, *Acta Chem Scand.* **6**, 611 (1952)

ing its early growth Arginine is apparently not required by the mouse for growth but is absolutely essential for the chick It can be synthesized by adult rats, dogs, or humans, but not at a rate compatible with optimal growth of young rats Citrulline can replace arginine in the diet of the rat and chick.[213]

Histidine is required for the growth of rat, dog, chick, and mouse, but

TABLE 2

AMINO ACID REQUIREMENTS OF SEVERAL SPECIES*

Amino acid	Dog and rat†[205 206]	Mouse†[207 208]	Chick†[209 210]	Man‡[211]
Alanine	–§	–	–	–
Arginine	+§	–	+	–
Aspartic acid	–	–	–	–
Cystine	–	–	(+)§	–
Glutamic acid	–	–	(+)	–
Glycine	–	–	(+)	–
Histidine	+	+	+	–
Hydroxyproline	–	–	–	–
Isoleucine	+	+	+	+
Leucine	+	+	+	+
Lysine	+	+	+	+
Methionine	+	+	+	+
Phenylalanine	+	+	+	+
Proline	–	–	(+)	–
Serine	–	–	–	–
Threonine	+	+	+	+
Tryptophan	+	+	+	+
Tyrosine	–	–	(+)	–
Valine	+	+	+	+

* Adapted from Albanese[212]
† Required for growth
‡ Required for nitrogen equilibrium
§ –, nonessential, +, essential, (+), required under certain conditions

not for the maintenance of nitrogen equilibrium in man.[214] Certain amino acids (e g , lysine, tryptophan, methionine, phenylalanine, leucine, valine, isoleucine, threonine) appear to be required by all species The ε-hydroxy analogue of lysine (α-amino-ε-hydroxy-n-caproic acid) cannot replace lysine in the diet of the rat [215] Rats fed a diet containing this lysine analogue or casein treated with nitrous acid develop an anemia which is reversed by

[213] A A. Klose and H J. Almquist, *J Biol. Chem* 135, 153 (1940)
[214] W C Rose, W J. Haines, D T Warner, and J E. Johnson, *J Biol Chem*. 188, 49 (1951)
[215] R Gingras, E Page, and R Gaudry, *Science* 105, 621 (1947).

"essential" amino acid, in addition to depending on the species, may be determined by the experimental criteria employed (e g growth, maintenance of nitrogen balance), the age of the animals, and also apparently on the presence of certain vitamins in the diet. The demonstration of the nonessential nature of a given amino acid under one set of experimental conditions does not preclude the possibility that it may be required under other physiological conditions, (e g. pregnancy, disease).

Although early studies suggested that tryptophan and lysine might be necessary for the growth of rats,[201] the classical work of Osborne and Mendel[202] on purified proteins represents the first demonstration of the essential nature of these amino acids Later, histidine[203] and cystine[204] were reported to be essential for the growth of rats These early studies were carried out with proteins deficient in certain amino acids. The discovery of methionine[57] in 1922 and of threonine[46] in 1936 made it possible for the first time to employ mixtures of purified amino acids instead of whole proteins in the diets of experimental animals. By the successive removal of one amino acid from the diet, it was possible to establish the dietary requirement of each amino acid.

As a result of the efforts of Rose and his collaborators, and other investigators, a rather complete picture of the amino acid requirements of several species is available.[205-212] A summary of some of these findings is given in Table 2

Several amino acids, (e g., alanine, aspartic acid, hydroxyproline, serine) can apparently be synthesized by all the animals. It is of interest that glycine, glutamic acid, and proline cannot be synthesized by the chick dur-

[201] E G. Willcock and F G Hopkins, *J. Physiol* 35, 88 (1906-1907)
[202] [illegible] 17, 325 (1914)
[203] [illegible]
[204] [illegible]
[205] [illegible] *nce* 86, 298 [illegible] Protein and [illegible] York, 1950, [illegible] C Thomas,
Springfield, Ill , 1951
[206] W. C. Rose and E E Rice, *Science* 90, 186 (1939).
[207] C D Bauer and C P Berg, *J Nutrition* 26, 51 (1943)
[illegible] 127, 375 (1939)
[illegible] 182, 541 (1950)

The failure of a given D-amino acid to support growth does not eliminate the possibility that it may be metabolized For example, D-leucine does not replace L-leucine in promoting the growth of rats However, isotopic studies have shown that the carbon chain of D-leucine is partly converted to L-leucine.[226] The ability of a D-amino acid to support growth therefore

TABLE 3

MINIMUM AND RECOMMENDED INTAKES FOR NORMAL MAN WHEN DIET FURNISHES SUFFICIENT NITROGEN FOR SYNTHESIS OF NON-ESSENTIALS*

(Strictly Tentative Values)

Amino acid	Minimum daily requirement, g	Recommended daily intake, g	Number of subjects tested
L-Tryptophan	0.25	0.5	31†
L-Phenylalanine	1.10	2.2	22
L-Lysine	0.80	1.6	27
L-Threonine	0.50	1.0	19
L-Valine	0.80	1.6	23
L-Methionine	1.10	2.2	13
L-Leucine	1.10	2.2	8
L-Isoleucine	0.70	1.4	8

* Data of Rose[221]

† All these subjects have been kept in balance on 0.3 g or less

TABLE 4

UTILIZATION OF D-AMINO ACIDS AND α KETO ACIDS FOR THE GROWTH OF RATS

Amino acid	D-Isomer	α Keto analogue
Arginine	− (227)*	
Histidine	+ (228)	+ (237)
Isoleucine	− (229)	+ (238)
Leucine	− (229)	+ (238)
Lysine	− (230)	
Methionine	+ (198, 231)	+ (239)
Phenylalanine	+ (232)	+ (240)
Threonine	− (233)	
Tryptophan	+ (234, 235, 235a)	+ (241)
Tyrosine	+ (236)	+ (240)
Valine	− + (229, 229a)	+ (242)

* Numbers in parentheses are reference numbers

[226] S Ratner, R Schoenheimer, and D Rittenberg, *J Biol Chem* 134, 653 (1940)
[227] A A Albanese, V Irby, and J E Frankston, *J Biol Chem* 160, 25 (1945)
[228] G J. Cox and C P Berg, *J Biol Chem* 107, 497 (1934)
[229] W C Rose, *Physiol Revs* 18, 109 (1938)
[229a] J White, W S Jones, and H A Sober, *J Biol Chem* 199, 505 (1952)
[230] C P Berg, *J Nutrition* 12, 671 (1936)

administration of lysine.[216, 217, 218] Tryptophan deficiency in rats is associated with ocular defects, anemia, and other disturbances.[219, 220, 221] The conversion of tryptophan to nicotinic acid is discussed in Section V.12. Methionine may be replaced by homocystine in the diet of rats, provided that "labile methyl" group donors or appropriate amounts of folic acid and vitamin B_{12} are included. L-Cystathionine is capable of replacing cystine in the diet of rats, and L-allocystathionine, which is converted to homocysteine, supports growth under the same conditions as does homocysteine.[222] (See Section V.14.)

Tyrosine has been found to exert a "sparing" action on phenylalanine, provided that sufficient amounts of the latter are included in the diet of chicks or rats.[209, 223, 224]

Rose[211] has found that eight amino acids are necessary for the maintenance of nitrogen balance in normal adult man. As a result of these studies, tentative quantitative values for the minimal requirements of the essential amino acids were suggested. The minimum daily requirements range from 0.25 g. for tryptophan to 1.1 g. for phenylalanine, methionine, and leucine (Table 3).

The amino acid requirements of human infants have been partially elucidated.[212] Thus isoleucine, leucine, methionine, valine, tryptophan, threonine, phenylalanine, and lysine are required for growth, and cystine and tyrosine are needed under some conditions. It is of interest that histidine and arginine are probably not required by the human infant for normal growth and nitrogen retention. Although arginine and histidine may not be required for nitrogen equilibrium in adults and infants, the possibility exists that a deficiency of these amino acids in the diet may lead to other abnormalities. For instance a dietary arginine deficiency has been reported to lead to decreased spermatogenesis in man.[225]

In most of the studies on the amino acid requirements of animals, it was necessary to use a number of racemic amino acids, owing to the lack of availability of large quantities of certain of the L forms. Specific study of the D forms indicates that several of these can be utilized for growth

216 A. G. Hogan, E. L. Powell, and R. E. Guerrant, *J. Biol. Chem.* 137, 41 (1941).

217 [illegible] *J.* 39, 203 (1945).

218 [illegible] (1947).

219 [illegible] *c* 97, 312 (1943).

220 [illegible]

221 J. R. Totter and P. L. Day, *J. Nutrition* 24, 159 (1942).

222 W. P. Anslow, Jr., S. Simmonds, and V. du Vigneaud, *J. Biol. Chem.* 166, 35 (1946).

223 M. Womack and W. C. Rose, *J. Biol. Chem.* 166, 429 (1946).

224 C. R. Grau, *J. Biol. Chem.* 170, 661 (1947).

225 L. E. Holt, Jr., A. A. Albanese, L. B. Shettles, C. Kajdi, and D. M. Wangerin, *Federation Proc.* 1, 116 (1942).

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L-Lysine	0 80	1 6	27
L-Threonine	0 50	1 0	19
L-Valine	0 80	1 6	23
L-Methionine	1 10	2 2	13
L-Leucine	1 10	2 2	8
L-Isoleucine	0 70	1 4	8

* Data of Rose [211]

† All these subjects have been kept in balance on 0 3 g or less

TABLE 4

UTILIZATION OF D-AMINO ACIDS AND α-KETO ACIDS FOR THE GROWTH OF RATS

Amino acid	D-Isomer	α Keto analogue
Arginine	− (227)*	
Histidine	+ (228)	+ (237)
Isoleucine	− (229)	+ (238)
Leucine	− (229)	+ (238)
Lysine	− (230)	
Methionine	+ (198, 231)	+ (239)
Phenylalanine	+ (232)	+ (240)
Threonine	− (233)	
Tryptophan	+ (234, 235, 235a)	+ (241)
Tyrosine	+ (236)	+ (240)
Valine	−, + (229, 229a)	+ (242)

* Numbers in parentheses are reference numbers

[226] S Ratner, R Schoenheimer, and D Rittenberg, *J Biol Chem* **134**, 653 (1940)

[227] A A Albanese, V Irby, and J E Frankston, *J Biol Chem* **160**, 25 (1945)

[228] G J Cox and C P Berg, *J Biol Chem* **107**, 497 (1934)

[229] W C Rose, *Physiol Revs* **18**, 109 (1938)

[229a] J White, W S Fones, and H A Sober, *J Biol Chem* **199**, 505 (1952)

[230] C P. Berg, *J Nutrition* **12**, 671 (1936)

depends upon the *in vivo* rate of conversion. The fact that α-ketoisocaproic acid promotes the growth of rats fed leucine-deficient rations at about the same rate as does L-leucine suggests that the limiting step in the utilization of D-leucine may be the rate of deamination. On the other hand, D-phenylalanine, D-methionine, D-tryptophan, and D-histidine have been reported to be capable of replacing the respective L isomers in supporting the growth of rats. Findings on the utilization of D isomers and the α-keto analogues of amino acids for the growth of rats are summarized in Table 4. All of the available α-keto analogues of the amino acids essential for rat growth can replace the respective amino acids. The preparation of the keto analogues of lysine, arginine, and L-threonine has not yet been reported. Although of the four stereoisomers of isoleucine only L-isoleucine supports the growth of rats, both the keto analogue of L-isoleucine and that of L-alloisoleucine are capable of promoting growth however not to an equal degree [238]

Although the growth of several laboratory animals and the maintenance of nitrogen balance in man can be achieved with a mixture of essential amino acids, there is evidence suggesting that these amino acids alone may not promote optimal growth. Rose *et al.*[243] have found that rats fed rations containing the ten essential amino acids gained only 70 to 75% as much weight as did their litter mates which received nineteen amino acids Observations on mice are in accord with this finding.[207, 212] It has been suggested that polypeptide fractions may be required for optimal growth[244] There is also evidence indicating that all the essential amino acids must be simultaneously available for protein synthesis. Studies by Cannon and his collaborators and other investigations of this problem have been recently reviewed [245]

In most studies on amino acid nutrition, the possible role of the intestinal flora has been neglected. The importance of intestinal organisms in

231 R W Jackson and R J Block, *Proc Soc Exptl Biol. Med* 30, 587 (1932–1933).
232 W. C Rose and M Womack, *J Biol Chem* 166, 103 (1946)
233 [illegible]
234 [illegible] 1932)
235 [illegible]
235a [illegible]
236 E C Bubl and J S Butts, *J Biol Chem* 174, 637 (1948)
237 B Harrow and C P Sherwin, *J Biol. Chem* 70, 683 (1926).
238 A Meister and J White, *J Biol Chem* 191, 211 (1951)
239 W M Cahill and G G Rudolph, *J. Biol Chem* 145, 201 (1942)
240 [illegible]
241 [illegible]
242 [illegible] 1 (1950).
243 [illegible] 753 (1948)
244 [illegible]
245 [illegible]

the synthesis of certain vitamins is well known Thus far, the few investigations in which antibacterial agents have been employed have not suggested that the intestinal flora plays a significant role [238-246, 247] Obviously germ-free animals will be required to answer this question unequivocally

The recent demonstration that rats fed ample amounts of folic acid and vitamin B_{12} were capable of growing on a diet deficient in methionine but containing homocystine (Section V 14) indicates again that the requirement for an amino acid is dependent upon the experimental conditions. It is conceivable that conditions may be found, which will permit growth in the absence of other "essential" amino acids

Inhibition of growth by certain amino acid derivatives has been observed. Although the exact mechanism by which these compounds act is not known, it appears probable that they interfere with the metabolism of a specific amino acid At least one of these derivatives (ethionine) may actually be incorporated into body protein [248] Although no attempt can be made here to discuss this subject extensively, several amino acid antagonists which have been studied in rats may be noted The effects of α-amino-ε-hydroxycaproic acid have been mentioned above Ethionine (α-amino-γ-ethiolbutyric acid), which does not replace methionine in promoting the growth of rats,[249] inhibits bacterial growth[250] and growth and protein synthesis in rats [248, 251, 252] A number of phenylalanine antagonists have been reported including β-2-thienylalanine,[253-256] β-3-thienylalanine,[257] and several fluorophenylalanine compounds [258] Penicillamine (β,β-dimethylcysteine) produced weight loss and death when fed to growing rats These effects were prevented by administration of aminoethanol or N-methyl derivatives of this compound [259]

[246] H L Williams and E M Watson, *Science* **103**, 654 (1946)
[247] W C Rose and L C Smith, *J Biol Chem* **187**, 687 (1950)
[248] M Levine and H Tarver, *J Biol Chem* **192**, 835 (1951)
[249] H M Dyer, *J Biol Chem* **124**, 519 (1938)
[250] R. O Roblin, Jr , J O Lampen, J P English, Q P Cole, and J R Vaughn, Jr , *J Am Chem Soc* **67**, 290 (1945)
[251] M V Simpson, E Farber, and H Tarver, *J Biol Chem* **182**, 81 (1950)
[252] J A Stekol and K Weiss, *J Biol Chem* **179**, 1049 (1949), **185**, 577 (1950)
[253] V du Vigneaud, H McKennis, Jr , S Simmonds, K Dittmer, and G. B Brown, *J Biol Chem* **159**, 385 (1945)
[254] K Dittmer, G Ellis, H McKennis, Jr , and V du Vigneaud, *J Biol Chem* **164**, 761 (1946)
[255] E Beerstecher, Jr , and W Shive, *J Biol Chem* **164**, 53 (1946)
[256] M F Ferger and V du Vigneaud, *J Biol Chem* **174**, 241 (1948), **179**, 61 (1949)
[257] R G Garst, E Campaigne, and H G Day, *J Biol Chem* **180**, 1013 (1949)
[258] M D Armstrong and J D Lewis, *J Biol Chem* **188**, 91 (1951), **190**, 461 (1951)
[259] J E Wilson and V du Vigneaud, *J Biol Chem* **184**, 63 (1950)

2. Other Organisms

The amino acid requirements of a number of bacteria have been determined and microbiological assays for the quantitation of most of the natural amino acids have been developed in the last eight years The lactic acid bacteria have been the most frequently employed for microbiological assays of amino acids. It is of interest that although assays have been developed for all the amino acids commonly found in proteins (Section II 1) apparently no requirement for hydroxyproline has yet been demonstrated The amino acid requirement of bacteria may be influenced by the conditions of growth, e.g., the composition of the medium, temperature, etc Furthermore some amino acids must be classified as "accessory" rather than "essential" or "non-essential" since they are not required for growth but increase the rate of growth. Other complicating factors include the possibility of "adapting" an organism to a given set of experimental conditions

These extensive researches have been reviewed recently by Snell[260] and Dunn,[261] and the reader is referred to their discussions for a detailed account of the history and development of this field. Kidder and Dewey[262] have recently reviewed the subject of the amino acid requirements of the *Tetrahymena*

IV. General Aspects of Amino Acid Metabolism

1 Nitrogen Metabolism

The major initial reaction in the breakdown of amino acids is loss of the α-amino group. Such a deamination may be due to oxidation or transamination The residual carbon skeleton may be re-aminated or converted to other products, while the α-amino group ultimately appears in mammals, mainly in urea, and in uric acid or allantoin in other species Obviously, the metabolic pathway is not the same for all the amino acids, nor can a single metabolic scheme describing the occurrence of these reactions in all species be elaborated However, certain general conclusions are possible

The important studies of Schoenheimer and his collaborators[226, 263-271]

260

261

262 emistry and Physiology of Protozoa, Academic Press, New York, 1951, Vol 1, p 323

263 S Ratner, D Rittenberg, A S Keston, and R Schoenheimer, *J Biol Chem* **134**, 665 (1940)

264 D Shemin and D. Rittenberg, *J Biol Chem* **153**, 401 (1944)

clearly demonstrated the dynamic state of body proteins and amino acids When amino acids or ammonia labeled with N^{15} were fed to rats, the animals subsequently sacrificed, and their tissues examined, the isotope was found in all but one (lysine) of the amino acids In all these experiments, glutamic and aspartic acids contained very high N^{15} concentrations Except in the case of the experiments with aspartic acid,[265] the amino acid fed had the highest N^{15} concentration, followed in order of isotope content by glutamic and aspartic acids In the studies in which aspartic acid was fed, glutamic acid had the highest N^{15} concentration It is significant that incorporation of isotope occurred even in rats on deficient diets leading to weight loss When the isotope was administered in the form of D-leucine, the incorporation of N^{15} into proteins was about the same as with L-leucine Using D-leucine labeled with deuterium, it was shown that some conversion of D-leucine to L-leucine occurs, although the failure of D-leucine to substitute for the L enantiomorph in promoting the growth of rats suggests that the formation of L-leucine is not sufficiently rapid to support growth. The finding of high concentrations of isotope in the dicarboxylic amino acids is in accord with the observation that these amino acids undergo rapid transamination and that ammonia is incorporated into glutamate via the glutamic dehydrogenase system The incorporation of N^{15} in the other amino acids may also be explained in terms of transaminase activity, since it is now recognized that this system has a wider scope than hitherto suspected (Section IV 5)

Lysine and threonine occupy a special position, in that these amino acids do not incorporate administered N^{15} When lysine labeled with deuterium and N^{15} was fed, N^{15} was found in other amino acids, but the lysine incorporated into tissues had the same $D.N^{15}$ ratio as the ingested lysine [270, 271] Similar results have been reported for threonine [272]

The end product of nitrogen metabolism in mammals is mainly urea, and it is generally accepted that this arises by the action of arginase on arginine to yield urea and ornithine In 1932, Krebs and Henseleit[273] proposed the

[264] H Wu and D Rittenberg, *J Biol Chem* **179**, 847 (1949).

[266] G L Foster, R Schoenheimer, and D Rittenberg, *J Biol Chem* **127**, 319 (1939)

[267] D Rittenberg, R Schoenheimer, and A S Keston, *J Biol Chem* **128**, 603 (1939)

[268] R Schoenheimer, S Ratner, and D Rittenberg, *J Biol Chem* **127**, 333 (1939)

[269] R Schoenheimer, S Ratner, and D Rittenberg, *J Biol Chem* **130**, 703 (1939).

[270] N Weissmann and R Schoenheimer, *J Biol Chem* **140**, 779 (1941)

[271] S Ratner, N Weissman and R Schoenheimer, *J Biol Chem* **147**, 549 (1943).

[272] D F Elliott and A Neuberger, *Biochem J* **46**, 207 (1950)

[273] H A Krebs and K Henseleit, *Z physiol Chem* **210**, 33 (1932)

2. Other Organisms

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The important studies of Schoenheimer and his collaborators[226, 263-271]

260 E E Snell, *Advances in Protein Chem* **2**, 85 (1945)

261 M S Dunn, *Physiol Revs* **29**, 219 (1949)

262 G. W Kidder and V C Dewey, *in* Lwoff, Biochemistry and Physiology of Protozoa, Academic Press, New York, 1951, Vol 1, p 323

263 S Ratner, D Rittenberg, A S Keston, and R Schoenheimer, *J. Biol Chem* **134**, 665 (1940)

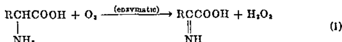
264 D Shemin and D Rittenberg, *J Biol Chem* **153**, 401 (1944).

the other hand it has been shown that leucine (Section V 4) phenylalanine, and tyrosine (Section V 13) lead to acetoacetate formation.

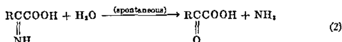
It is apparent that conclusive evidence on the fate of the carbon moieties of amino acids can be obtained only in studies in which the amino acid is labeled, and where the label is traced in the products of metabolism A number of such studies have been carried out, and some of these are described in Section V

3 Deamination by the General Amino Acid Oxidases

a. D-Amino Acid Oxidase. ly studies suggested that biological deamination of amino acids inv ed oxidation to the corresponding keto acid[287, 288] However, the first finitive studies on amino acid oxidation were carried out by Krebs[29] [90] [291] The oxidation of amino acids is believed to proceed as follows.

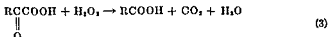


$$\underset{\displaystyle NH_2}{RCHCOOH} + O_2 \xrightarrow{\text{(enzymatic)}} \underset{\displaystyle \| \atop NH}{RCCOOH} + H_2O_2 \quad (1)$$



$$\underset{\displaystyle \| \atop NH}{RCCOOH} + H_2O \xrightarrow{\text{(spontaneous)}} \underset{\displaystyle \| \atop O}{RCCOOH} + NH_3 \quad (2)$$

In the presence of catalase, the products are the corresponding α-keto acid and ammonia. With systems in which catalase is lacking, the α-keto acid undergoes further non-enzymatic oxidation

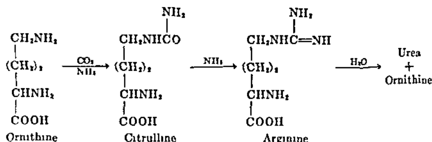


$$\underset{\displaystyle \| \atop O}{RCCOOH} + H_2O_2 \rightarrow RCOOH + CO_2 + H_2O \quad (3)$$

Krebs found that cell-free preparations of kidney were capable of oxidizing many D-amino acids in accordance with equations 1 and 2 The D-amino acid oxidase was readily distinguished and separated from the system responsible for the oxidation of L-amino acids. Subsequent research has shown that D-amino acid oxidase possesses a coenzyme, flavin adenine dinucleotide[292] [293] [294] The variations in specificity of the enzyme in different species may be attributed to the nature of the protein apoenzyme Since the original work of Krebs, a large body of data on the distribution, speci-

287 O Neubauer, *Deut Arch Klin Med* **95**, 211 (1909)
288 F Knoop, *Z. physiol Chem* **67**, 489 (1910).
289 H. A Krebs, *Klin Wochschr* **11**, 1744 (1932)
290 H. A Krebs, *Z physiol Chem* **217**, 191 (1933)
291 H. A. Krebs, *Biochem J* **29**, 1620 (1935).
292 O. Warburg and W Christian, *Biochem Z* **296**, 294 (1938), **298**, 150 (1938)
293 F. B Straub, *Nature* **141**, 603 (1938); **143**, 76 (1938).
294 F B Straub, *Biochem J.* **33**, 787 (1939).

following mechanism for the formation of urea:



Although citrulline formation has been demonstrated,[274] it is now clear that the formation of urea is more complicated than indicated in this "cycle" (see Section V 9).

2 Carbon Metabolism

The deamination of α-amino acids—whether by oxidation or transamination—leads to the formation of the corresponding α-keto acids The keto acid may be reaminated and incorporated into protein or may be degraded, ultimately yielding carbon dioxide and water Under suitable experimental conditions, it can be demonstrated that certain amino acids lead to an increased formation of liver glycogen or glucose, while others produce an increase in acetic, acetoacetic, or β-hydroxybutyric acid formation. The classification of amino acids as glycogenic or ketogenic resulted from observations of this nature It is obvious that deamination of alanine, aspartic acid, and glutamic acid yields keto acids which are intermediates in the metabolism of carbohydrates These amino acids, as well as valine,[275, 276] serine,[277, 278] glycine,[277, 279] threonine,[280] arginine,[281, 282] histidine,[282, 283, 284] and isoleucine,[282, 285] have been reported to produce an increase in liver glycogen in fasted animals "Extra" carbohydrate is also formed, under appropriate conditions, from methionine,[286] cysteine,[282] and proline [282] On

[274] A G. Gornall and A Hunter, *J Biol Chem* **147**, 593 (1943)
[275] W C Rose, J E Johnson, and W J Haines, *J Biol Chem* **145**, 679 (1942).
[276] J S Butts and R O Sinnhuber, *J Biol Chem* **139**, 963 (1941)
[277] F A Schofield and H B. Lewis, *J Biol. Chem* **169**, 373 (1947).
[278] J S Butts, H Blunden, and M S Dunn, *J Biol. Chem* **124**, 709 (1938)
[279] A J Ringer and G Lusk, *Z physiol Chem* **66**, 106 (1910)
[280] W. K. Hall, J R Doty, and A G Eaton, *Am J Physiol* **131**, 252 (1940), **129**, 572 (1940).
[281] [illegible] (1941)
[282] [illegible]
[283] [illegible] 42)
[284] [illegible] (1942)
[285] [illegible] 20, 289 (1937).
[286] [illegible]

Data taken from the literature are given in Table 5 In addition to the substrates listed in Table 5, D-amino acid oxidase attacks hydroxy-D-proline,[306] allohydroxy-D-proline,[306] D-alloisoleucine,[307] D-allothreonine,[187] certain N-monomethyl-D-amino acids,[291, 308] S-alkyl-D-cysteine derivative,[200] D ethionine,[200] D-α-aminophenylacetic acid,[193] D-cyclohexyl-α-aminoacetic acid,[193] D-β-cyclohexyl-α-aminopropionic acid,[193] D-β-2-thienylalanine,[309] and number of other substrates [299, 309, 310]

The enzyme does not attack amino acids in which (a) the α-hydrogen atom is substituted or (b) both amino group hydrogen atoms are substituted

The physiological role of D-amino acid oxidase is as yet unknown It has been suggested that this enzyme may (a) be an artifact produced in its preparation from tissue, (b) metabolize naturally occurring D-amino acids which may enter the body, and (c) destroy D-amino acids formed in the body by symmetrical amino acid synthesis The last possibility appears unlikely [311] In experiments in which racemic nitrogen-labeled tyrosine and glutamic acid were administered to animals, there was no dilution of the D-amino acid excreted in the urine However, this result does not provide an unequivocal solution to the problem, since the administered amino acid might not have penetrated into the cells or have reached the active enzyme surface

b. L-Amino Acid Oxidase. (1) *Rat Kidney Enzyme* Green and coworkers[304] isolated an amino acid oxidase from rat kidney which catalyzed the oxidation of thirteen L-amino acids in accordance with equations 1 and 2 (Table 5) The purified enzyme was also found to oxidize certain α-hydroxy acids This enzyme also differs from kidney D-amino acid oxidase in that it possesses riboflavin phosphate as its prosthetic group It exhibits an extremely low turnover number (6 as compared to 1440 for the D-oxidase of Krebs) [296] Because of the low activity, limited specificity, and restricted distribution of this system, it appears improbable that it alone is responsible for the breakdown of L-amino acids in mammalian tissues

(2) *Ophio-L-Amino Acid Oxidase* The presence of L-amino acid oxidase

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[302] M D Webster and F Bernheim, *J Biol Chem* **114**, 265 (1936)
[303] E A Zeller and A Maritz, *Helv Chim Acta* **27**, 1888 (1944), **28**, 365 (1945).
[304] M Blanchard, D E Green, V Nocito, and S Ratner, *J Biol Chem* **155**, 421 (1944), **161**, 583 (1945)
[305] P K Stumpf, and D E Green, *J Biol Chem* **153**, 387 (1944)
[306] D S Robinson and J P Greenstein, *J Biol Chem* **195**, 383 (1952)
[307] A Meister, *J Biol Chem* **190**, 269 (1951)
[308] P Handler, F Bernheim, and J R Klein, *J Biol Chem* **138**, 203 (1941)
[309] E Frieden, L T Hsu, and K Dittmer, *J Biol Chem* **192**, 425 (1950)
[310] A Meister, *J Biol Chem* **197**, 309 (1952)
[310a] B M Brabanca and J N Quastel, *Arch Biochem Biophys* **40**, 130 (1952)
[311] D Shemin and D Rittenberg, *J Biol Chem* **151**, 507 (1943)

ficity, coenzyme, and purification[295] of D-amino acid oxidase has accumulated [296] The enzyme has been found in the kidney and liver of almost all species investigated,[297] as well as in certain molds[298, 300] and bacteria [301, 302]

TABLE 5

OXIDATION OF AMINO ACIDS

Amino acid	D-Amino acid oxidase: Sheep kidney*	D-Amino acid oxidase: *N crassa**	L-Amino acid oxidase: *Vipera aspis* venom†	L-Amino acid oxidase: *N. crassa**	L-Amino acid oxidase: Rat kidney‡	L-Amino acid oxidase: *Proteus vulgaris*§
	qO_2	qO_2	qO_2	qO_2		
Alanine	64	0 8	30	41		0
α-Aminobutyric acid	31	3 0		64	3	12
Norvaline	20	2 2		75	53	60
Norleucine	36	0 8		60	89	108
α-Aminocaprylic acid	1 9	Nil		17		
Valine	35	0 2	71	6	28	0
Leucine	13 9	1.5	612	78	(100)	91
Isoleucine	22	0.2	162	33	71	15
Aspartic acid	*1 4*	*0 3*		*5*	*0*	*0*
Glutamic acid	Nil	0 7		9	0	0
αAminoadipic acid	Nil	0 2		61		
Phenylalanine	26	1 2	780	41	45	(100)
Tyrosine	190	0 3	402	24	20	62
Histidine	6 2	0 5	78	37	9	33
Tryptophan	37	Nil	540	27	40	88
Lysine	0 6	Nil		14	0	0
Ornithine	3 1	Nil		51	0	0
Serine	42	Nil		8	0	0
Threonine	*2 1*	*Nil*		*2*	*0*	*0*
Proline	148	Nil		Nil	77	0
Methionine	80	3 0	390	40	81	65
Cystine	1 9	Nil	42	56	15	
Arginine			26			30

* Data of Bender and Krebs [299]
† Data of Zeller and Maritz [303]
‡ Data of Blanchard, *et al* [304] Values given as per cent of rate with leucine
§ Data of Stumpf and Green [305] Values given as per cent of rate with DL-phenylalanine

295 [illegible]

296 [illegible] Cambridge University

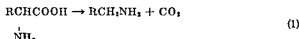
297 It is apparently absent in mouse liver; J Shack, *J. Natl Cancer Inst* **3**, 389 (1943)

298 N. H. Horowitz, *J. Biol Chem* **154**, 141 (1944)

[illegible] *hem* **25**, 299 (1950).

[illegible] *Biol Chem.* **110**, 165 (1935).

acids according to the equation:



$$\underset{\displaystyle NH_2}{RCHCOOH} \rightarrow RCH_2NH_2 + CO_2 \qquad (1)$$

A summary of the amino acid decarboxylases which have thus far been discovered is given in Table 6. The first of the decarboxylases of animal

TABLE 6

AMINO ACID DECARBOXYLASES

Susceptible amino acid	Product of decarboxylation	Sources of decarboxylase
Arginine	Agmatine	Bacteria[322, 323]
Aspartic acid	β-Alanine	Bacteria[324, 325]
Aspartic acid	α-Alanine	*Clostridium welchii*[326]
Cysteic acid	Taurine	Mammalian tissues[327]
α,ε-Diaminopimelic acid	Lysine	Bacteria[350b]
3,4-Dihydroxyphenylalanine	3,4-Dihydroxyphenylethylamine	Mammalian tissues[328, 329] Bacteria[322, 330]
Glutamic acid	γ-Aminobutyric acid	Bacteria[322, 331] Plants[332, 333, 334] Mammalian tissues[335, 336]
Histidine	Histamine	Mammalian tissues[337, 338]
p-Hydroxyphenylserine	p-Hydroxyphenylethanolamine	Mammalian tissues[339]
Lysine*	Cadaverine	Bacteria[322, 340]
Ornithine	Putrescine	Bacteria[322]
Phenylalanine	Phenylethylamine	*Streptococcus faecalis*[341]
Tyrosine	Tyramine	Mammalian tissues[342, 322] Bacteria[322]
Tryptophan	Tryptamine	Mammalian tissues[343, 344]

* Lysine decarboxylase has been reported to decarboxylate hydroxylysine[340a]

[322] E F Gale, *Biochem J* **34**, 392, 846, 853 (1940), **35**, 66 (1941)
[323] E S Taylor and E F Gale, *Biochem J* **39**, 52 (1945)
[324] D Billen and H C Lichstein, *J Bact* **58**, 215 (1949)
[325] W E Davis and H C Lichstein, *Proc Soc Exptl Biol Med* **73**, 216 (1950)
[326] A Meister, H A Sober and S V Tice, *J Biol Chem* **189**, 577, 591 (1951)
[327] H Blaschko, *Biochem J* **36**, 571 (1942)
[328] P Holtz, R Heise, and K Lüdtke, *Arch Exptl Path Pharmakol* **191**, 87 (1938)
[329] P Holtz and K Credner, *Arch Exptl Path Pharmakol* **199**, 145 (1942)
[330] H M R Epps, *Biochem J* **38**, 242 (1944)
[331] E F Gale, *Advances in Enzymol* **6**, 1 (1946)
[332] K Okunuki, *Botan Mag (Tokyo)* **51**, 270 (1937)
[333] O Schales, V Mimms and S S Schales, *Arch Biochem* **10**, 455 (1946).
[334] O Schales and S S Schales, *Arch Biochem* **11**, 155 (1946)
[335] W J Wingo and J Awapara, *J Biol Chem* **187**, 267 (1950)
[336] E Roberts and S Frankel, *J Biol Chem* **188**, 789 (1951), **190**, 505 (1951)
[337] E Werle, *Biochem Z* **288**, 292 (1936)

in the venoms and tissues of various snakes was first reported by Zeller and his collaborators[303, 312, 313] Oxidation of a wide variety of amino acids takes place in accordance with equations 1, 2, and 3 unless catalase is added to the system In addition to the susceptible substrates listed in Table 5, preparations of venom oxidize L-alloisoleucine,[314] L-asparagine,[312] L-glutamine,[312] the γ-methylamide, γ-ethylamide, and γ-ethyl ester of L-glutamic acid,[310] ε-benzoyl-L-lysine,[303] ε-chloroacetyl-L-lysine,[310] L-citrulline,[303] L-cyclohexyl-α-aminoacetic acid,[193] L-α-aminophenylacetic acid,[193] L-β-cyclohexyl-α-aminopropionic acid,[193] certain higher homologues of L-alanine,[299] δ-chloroacetyl-L-ornithine,[315] 3,5-diiodo-L-tyrosine,[312] and a number of other substrates[299, 309] The enzyme does not attack N-monomethyl-L-amino acids,[312] DL-β,β,β-trimethylalanine,[316] L-proline,[312] hydroxy-L-proline,[306] or α-aminoisobutyric acid.[316] It has been suggested that the β-hydrogen plays an important role in the deamination of amino acids.[316] However, the D and L isomers of α-aminophenylacetic acid were found to be oxidized by hog kidney and venom preparations, respectively.[200] It is noteworthy that oxidation of the four stereoisomers of isoleucine by kidney and venom enzymes leads to the formation of the respective optical isomers of α-keto-β-methylvaleric acid, indicating that an α,β unsaturation leading to a loss of configuration at the β-carbon atom does not occur.[307, 314] The enzymatic oxidation of phenylserine isomers to the corresponding optically active mandelic acids provides additional evidence on this point.[317]

Ophio-L-amino acid oxidase has been purified and shown to possess flavin adenine dinucleotide as a prosthetic group.[318]

(3) *L-Amino Acid Oxidase of Molds and Bacteria.* The oxidation of L-amino acids by preparations of certain molds[299, 319, 320, 321] and bacteria[305] has been reported Representative data are given in Table 5 It is of interest that the formation of the oxidase by *Neurospora crassa* is stimulated by biotin[321]

4 Decarboxylation of Amino Acids

It is now recognized that certain animal, plant, and bacterial systems are capable of catalyzing the decarboxylation of a number of L-amino

312 [illegible] 1615 (1915)

313 [illegible]

314 [illegible]

315 [illegible]

316 G. A Fleisher and A E Zeller, *Abstracts*, San Francisco Meeting, American Chemical Society, p 20C (April, 1949)

317 W. S. Fones, *Arch Biochem Biophys* 136, 486 (1952)

318 [illegible] *Biochem* 27, 348 (1950); 29, 190 (1950)

319 [illegible]

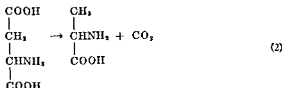
320 [illegible] I Horowitz, *Biochem. J* 45, xxi (1949)

321 P. S Thayer and N H Horowitz, *J Biol. Chem* 192, 755 (1951)

An important advance was the application of these systems to the quantitative determination of amino acids These studies have been reviewed recently by Gale [331]

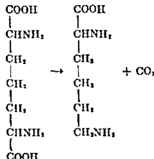
In addition to the decarboxylases investigated by Gale, it was found by McGilvery and Cohen[341] that *Streptococcus faecalis* preparations were capable of decarboxylating phenylalanine as well as tyrosine Both substrates may be metabolized by the same enzyme, although tyrosine is more susceptible The decarboxylation of aspartic acid to β-alanine has been investigated in several organisms [324, 325, 349, 350] In some cases the extremely low activity may be detected only by microbiological determination of the formation of β-alanine

Meister, Sober, and Tice[326] have described the decarboxylation of aspartic acid to α-alanine by *Clostridium welchii* This decarboxylation system (which should properly be designated as an aspartic-β-decarboxylase to distinguish it from the systems responsible for β-alanine formation from aspartic acid) is unique in that the β-carboxyl group is attacked and that the product of the reaction is an α-amino acid.



A similar system may exist in other organisms [350a]

Another decarboxylase system which yields an α-amino acid has been recently described by Dewey and Work [350b] These investigators demonstrated the decarboxylation of α,ε-diaminopimelic acid to L-lysine by a bacterial system:



The reaction is of significance in the synthesis of lysine in *E coli* [350c]

[350c] B D Davis, *Nature* 169, 537 (1952)

origin, histidine decarboxylase, was discovered by Werle[339] in 1936 Most of the decarboxylases of animal origin are found chiefly in the kidney, exhibit optimum activity at neutral or alkaline values of pH, and are reversibly inhibited by cyanide and carbonyl reagents Although the function of the amino acid decarboxylases of animal tissues is not completely understood, it would appear that decarboxylation is not a major general pathway of amino acid metabolism Decarboxylation reactions are probably important for the formation of certain compounds such as adrenaline and histamine [338]

An interesting recent development in this field was the independent discovery in three laboratories of the presence of γ-aminobutyric acid in brain tissue,[62, 63, 64] and the subsequent finding of a glutamic decarboxylase in brain and possibly other mammalian tissues.[335, 336] In view of the great interest in this problem, rapid advancement of our knowledge of the metabolism and role of γ-aminobutyric acid may be expected.

Okunuki[332] and Schales *et al* [333, 334] have studied the decarboxylation of glutamic acid by a variety of plant tissues, and the latter investigators have studied the kinetics of the enzyme system obtained from carrots It is possible that other amino acid decarboxylases may exist in plant tissues (cf ref 344)

The study of the bacterial amino acid decarboxylases was first intensively pursued by Gale [322, 331, 340] (See refs 345–348 for earlier work on amine formation) In contrast to the systems of animal origin, the bacterial enzymes exhibit optimal activity at acid values of pH. Investigations have been made of the formation, distribution, kinetics, and purification of the lysine, ornithine, histidine, tyrosine, glutamic acid, and arginine decarboxylases

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338 See H Blascho, *Advances in Enzymol* **5**, 67 (1945), for a review of the literature on amino acid decarboxylases of mammalian tissues

339 E Werle, *Z Vitamin , Hormon- und Fermentforsch* **1**, 504 (1947–1948)

340 E F Gale and H M R Epps, *Biochem J* **38**, 232 (1944)

341 R W McGilvery and P P Cohen, *J Biol Chem* **174**, 813 (1948)

342 P Holtz and H Janish, *Arch Exptl Path Pharmakol* **186**, 684 (1937)

343 E Werle and G Menniken, *Biochem Z* **291**, 325 (1937)

343a S Linstedt, *Acta Chem Scand* **5**, 486 (1951)

344 E Werle and A Raub, *Biochem Z* **318**, 538 (1948).

345 A Ellinger, *Ber* **31**, 3183 (1898), *Z. physiol Chem* **29**, 334 (1900)

346 W Barger and G S Walpole, *J Physiol* **38**, 343 (1909)

347 D Ackermann, *Z physiol Chem* **56**, 305 (1908), **65**, 504 (1910), **69**, 273 (1910)

348 M T Hanke and K K Koessler, *J Biol Chem* **39**, 539 (1919), **50**, 131 (1922), **59**, 835, 855, 867 (1924)

349 A I Virtanen and T Laine, *Enzymologia* **3**, 266 (1937)

350 A I Virtanen, P Rintola, and T Laine, *Nature* **142**, 674 (1938)

350a S R Mardashev, L A Semina, R N Etinhof, and A I Baliasnaia, *Biokhimiya* **14**(1), 44 (1949)

350b D L. Dewey and E Work, *Nature* **169**, 533 (1952).

actions were:



Glutamate + oxaloacetate $\rightleftharpoons$ α Ketoglutarate + aspartate (2)



Glutamate + pyruvate $\rightleftharpoons$ α Ketoglutarate + alanine (3)



Aspartate + pyruvate $\rightleftharpoons$ Oxaloacetate + alanine (4)

Cohen, at this time, considered transamination to be limited to the reactants given in equations 2, 3, and 4 Braunstein essentially accepted this point of view, indicating that the analytical methods originally employed were not adequate [354]

In 1942, Lenard and Straub[360] obtained a purified glutamic-alanine transaminase. Subsequently active preparations of glutamic-alanine[361] and glutamic-aspartic[361, 362, 363, 363a] transaminases have been described The glutamic-aspartic transaminase preparation of Green *et al* was shown to contain codecarboxylase (see below)

The question of the existence of a separate aspartic-alanine system remains in doubt Green *et al* [361] suggested that this system might represent an artifact, owing to a mixture of glutamic-aspartic and glutamic-alanine transaminases, and this concept was supported by the experimental findings of O'Kane and Gunsalus [363] Moulder *et al* [364] observed carbon dioxide formation from aspartate in pigeon liver preparations, which was catalyzed by pyruvate and Mn^{++} Although they interpreted the findings in terms of an aspartic-pyruvate transamination, followed by oxaloacetate decarboxylation, the possibility of a direct β-carboxylation of aspartate was not excluded (cf ref 326) Kritsmann and Samarina have presented evidence for an aspartic-pyruvic system in liver [365] It is obvious that conclusions based on studies with pig heart muscle systems may not apply to the liver As discussed below, the liver is known to contain at least two transaminase systems not found in heart muscle

Although transamination was considered to be limited to aspartic acid, glutamic acid, alanine, and their α-keto analogues, less rapid transamination reactions involving leucine, methionine, isoleucine, valine, cysteic acid, phenylalanine, and tyrosine had been demonstrated in relatively early studies [354, 366] More recently, independent studies by Tannenbaum and

[360] P Leonard and F B Straub, *Studies Inst Med Chem Univ Szeged* **2**, 59 (1942)
[361] D E Green, L F Leloir, and V Nocito, *J Biol Chem* **161**, 559 (1945)
[362] F Schlenk and A. Fisher, *Arch Biochem* **12**, 69 (1947)
[363] D O'Kane and I C Gunsalus, *J Biol Chem* **170**, 425, 433 (1947)
[363a] P S Cammarata and P P Cohen, *J Biol Chem* **193**, 53 (1951)
[364] J W Moulder, B Vennesland, and E A Evans, Jr, *J Biol Chem*, **160**, 305 (1945).
[365] M G Kritsmann and O P. Samarina, *C A* **43**, 2252 (1949)
[366] P P. Cohen, *J Biol Chem*, **133**, *Proc* xx (1940)

The coenzyme for the amino acid decarboxylases is believed to be pyridoxal-5-phosphate. The *requirement for preparations of this derivative by* decarboxylase systems has been demonstrated for many of the amino acid decarboxylases Although it seems probable that pyridoxal phosphate may be involved in all these systems, efforts to demonstrate its function in certain systems have thus far been unsuccessful Pyridoxal phosphate is considered in detail in the following section.

5 Transamination

In 1930, Needham[351] observed the anaerobic disappearance of glutamate and aspartate in pigeon breast muscle She was unable to find an associated increase in urea or ammonia, and considered the possibility of the formation of a new amino acid Six years later it was reported by Annau *et al.*[352] that the disappearance of oxaloacetate in pigeon breast muscle was accelerated by glutamate. In 1937, Braunstein and Kritsmann[353, 354, 355] reported the first detailed description of a biological transamination reaction. These *workers described a system in muscle capable of reversibly transferring* the amino group of glutamic acid to pyruvic acid:

$$\begin{array}{l} \text{COOH} \\ | \\ \text{CH}_2 \\ | \\ \text{CH}_2 \\ | \\ \text{CHNH}_2 \\ | \\ \text{COOH} \end{array} + \begin{array}{c} \text{CH}_3\text{CCOOH} \\ \| \\ \text{O} \end{array} \rightleftharpoons \begin{array}{l} \text{COOH} \\ | \\ \text{CH}_2 \\ | \\ \text{CH}_2 \\ | \\ \text{C}{=}\text{O} \\ | \\ \text{COOH} \end{array} + \begin{array}{c} \text{CH}_3\text{CHCOOH} \\ | \\ \text{NH}_2 \end{array} \qquad (1)$$

This reaction did not lead to free ammonia formation, proceeded anaerobically, and was specific for the L isomers of the amino acids Originally the Russian workers reported that oxaloacetic acid and α-ketoglutaric acid were capable of transaminating with a large number of α-amino acids (except glycine) Cohen[356-359] reported that the principle transaminase re-

ki, F B Straub, and A

2, 129 (1937)

47)

3, 603 (1938).

[357] P P Cohen, *J Biol Chem* 136, 565, 585 (1940)

[358] Enzymes, University of Wisconsin

[359] *Chem* 140, 711 (1941)

dicarboxylic keto acids is compatible with this hypothesis.[372a] It is of interest that Cavallini and de Marco[372b] observed that the synthesis of phenylalanine and tyrosine from the respective α-keto acids in kidney preparations was greater in the presence of glutamine or asparagine than in the presence of glutamate or aspartate.

A number of bacteria can utilize the α-keto analogues of certain amino acids in place of the amino acids, provided that high levels of vitamin B_6 are included in the medium. The available evidence indicates that the utilization of α-keto acids for the growth of bacteria is dependent upon transamination to the corresponding amino acid.[373]

The existence of several separate apotransaminases, different from the two classical transaminases, is suggested by recent studies in which *Escherichia coli* extracts were fractionated.[374] One of these systems catalyzes transamination between glutamate, aspartate, phenylalanine, tyrosine, and tryptophan; the other catalyzes reactions between glutamate, valine, isoleucine, leucine, norleucine, and norvaline.

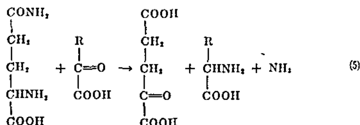
Studies employing deuterium-labeled alanine and glutamic acid indicated that the α-deuterium atom of the amino acid is labilized during transamination.[375, 376] The labilization of the α-deuterium atom of alanine (but not of glutamic acid) requires the presence of an α-keto acid. It is of interest that the loss of α-deuterium also occurs with boiled enzyme. The evidence is compatible with complex formation between the α-amino group and the coenzyme or enzyme.

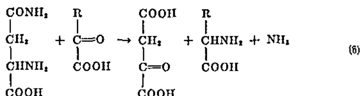
Further evidence of the wide scope of transamination is the finding that L-alloisoleucine, L-cyclohexylalanine, L-phenylglycine, L-ethionine, and other unnatural amino acids transaminate with α-ketoglutarate.[377] The configuration of the β-carbon of isoleucine is maintained after transamination.[314, 377]

There is now little doubt that transamination takes place between monocarboxylic amino and keto acids, although it was previously assumed that glutamate (or α-ketoglutarate) was a necessary participant in all transamination reactions. In early studies good analytical procedures for many amino and keto acids were not available, and the occurrence of other types of transamination could not properly be evaluated. Recent studies indicate that glutamine,[369] asparagine[372] and α-ketosuccinamic acid[372a] participate

[372a] A. Meister, *J. Biol. Chem.* 200, 571 (1953).
[372b] D. Cavallini, and C. De Marco, *Atti reale accad. Naz. Lincei*, Ser. VIII, 9, (6), 374 (1950).
[373] J. T. Holden, R. B. Wildman, and E. E. Snell, *J. Biol. Chem.* 191, 599 (1951).
[374] D. Rudman and A. Meister, *J. Biol. Chem.* 200, 591 (1953).
[375] A. S. Konikova, M. G. Kritsmann, and R. V. Teiss, *Biokhimiya* 7, 86 (1942).
[376] A. S. Konikova, N. N. Dobbert, and A. E. Braunstein, *Nature* 159, 67 (1947).
[377] A. Meister, *J. Biol. Chem.* 195, 813 (1952).

Shemin,[367] Meister and Tice,[368] Cammarata and Cohen,[369] and Feldman and Gunsalus[370] have clearly demonstrated the wide scope of enzymatic transamination It is now established that a large number of amino acids are capable of transaminating with α-ketoglutaric acid to yield glutamic acid [367, 369, 370, 371] Furthermore, fractions obtained from liver have been found to catalyze the following transamination-deamidation reactions involving glutamine,[368] asparagine,[372] and a wide variety of α-keto acids:


(5)


(6)

Experiments with N^{15}-labeled glutamine indicate that the ammonia formed is derived entirely from the amide group and not from the α-amino group Substitution of the amino acid ω-amide by the corresponding dicarboxylic amino acid (except in the cases of pyruvate and α-ketobutyrate) leads to loss of activity or a considerable reduction in transaminase activity. Since no deamidation occurs in the absence of α-keto acids, these studies would appear to indicate a close relationship between the processes of transamination and deamidation It was suggested that the α-keto analogues of glutamine (α-ketoglutaramic acid) and asparagine (α-ketosuccinamic acid) might be intermediates in these reactions [368, 372] The finding that these α-keto acid ω-amides are hydrolyzed to ammonia and the corresponding

[367] S. Tannebaum and D. Shemin, *Federation Proc.* 9, 236 (1950).
[368] A Meister, *Federation Proc* 9, 204 (1950), A Meister, and S V. Tice, *J. Biol Chem.* 187, 173 (1950).

ol.

319

(1952).

transamination was the finding by Schlenk and Snell[382] in 1945 that the tissues of vitamin B_6-deficient rats exhibited a low order of glutamic-aspartic transaminase activity This finding was confirmed in rats by Ames *et al*[383] in 1947. Later the glutamic-aspartic system of vitamin B_6-deficient hamsters[384] and the glutamic-pyruvic system (but not the glutamine-keto acid system) of vitamin B_6-deficient rats[385] were found to be reduced in activity. Lichstein *et al*[386] in 1945 found that *Streptococcus faecalis* R cells, grown on vitamin B_6-deficient media, exhibited a markedly reduced transaminase activity, which was greatly increased on addition of pyridoxal phosphate In the studies with vitamin B_6-deficient rats it was found that addition to the tissue extracts of pyridoxal and adenosine triphosphate resulted in an increase in transaminase activity

In 1943 Bellamy and Gunsalus[387, 388, 389] had observed a relationship between the activity of the tyrosine decarboxylase of *S faecalis* and the concentration of pyridoxine in the medium *S faecalis* cells grown on a medium deficient in vitamin B_6 exhibited little or no decarboxylase activity, whereas the addition of pyridoxal restored activity Using dried cells, these workers found that activity was restored by adenosine triphosphate plus pyridoxal, or a crude synthetic pyridoxal phosphate preparation Incubation of the cells (at pH 7 0) with pyridoxamine and pyruvate also led to "codecarboxylase" formation as indicated by restoration of tyrosine decarboxylase activity[390]

[illegible]

remains unsettled Of particular interest is the observation of Umbreit *et al*,[391] that, although impure preparations of pyridoxamine phosphate and pyridoxal phosphate activated crude preparations of apotransaminase, only pyridoxal phosphate was active with a highly purified pig heart transaminase Recently activation of the purified pig heart glutamic-aspartic apotransaminase by crystalline pyridoxamine phosphate was reported It

[382] F Schlenk and E E Snell, *J Biol Chem* **157**, 425 (1945)

[383] S R Ames, P S Sarma, and C A Elvehjem, *J Biol Chem* **167**, 135 (1947)

[384] G Shwartzman and H Hift, *J Nutrition* **10**, 575 (1951)

[385] A Meister, H P Morris, and S V Tice, *Proc Soc Exptl Biol Med*, **82**, 301 (1953)

[386] H C Lichstein, I. C Gunsalus, and W W Umbreit, *J Biol Chem* **161**, 311 (1945)

[387] W D Bellamy and I C Gunsalus, *J Bact* **46**, 573 (1943), **48**, 191 (1944), **50**, 95 (1945)

[388] I C Gunsalus and W. D Bellamy, *J Bact* **47**, 413 (1944)

[389] I C Gunsalus and W D Bellamy, *J Biol Chem* **155**, 35, 557 (1944)

[390] I C Gunsalus, W. D Bellamy, and W W Umbreit, *J Biol Chem* **155**, 685 (1944)

[391] W W Umbreit, O S O'Kane, and I C. Gunsalus, *J Biol Chem* **176**, 629 (1948)

(without prior deamidation) in transamination Preliminary reports describing an ornithine-pyruvate system[378] and transmination between several monocarboxylic amino and α-keto acids[379] have appeared. Reversible transamination between alanine (or α-aminobutyrate) and α-ketoisovalerate has been demonstrated in an *E. coli* mutant genetically blocked in transamination between valine and other amino acids including glutamate The responsible enzyme was separated by selective elution from calcium phosphate gel[374] The demonstration of the wide scope of transamination

to careful fractionation, assays involving all possible substrate combinations

In 1944, Snell[180] suggested that reversible transformations between pyridoxal and pyridoxamine might be involved in transamination and found that heating pyridoxamine with α-ketoglutarate resulted in pyridoxal and glutamate formation[381] The reaction was reversible It was suggested by Schlenk and Fisher[167] that enzymatic transamination involved the following intermediate reactions

$$\mathrm{\overset{COOH}{\underset{R_1}{C{=}O}} + H_2NH_2C{-}\langle N \rangle \; \underset{+H_2O}{\overset{-H_2O}{\rightleftharpoons}} \; \overset{COOH}{\underset{R_1}{C{=}N{-}H_2C}}{-}\langle N \rangle}$$

$$\mathrm{\overset{COOH}{\underset{R_1}{HC{-}NH_2}} + OHC{-}\langle N \rangle \; \underset{+H_2O}{\overset{-H_2O}{\rightleftharpoons}} \; \overset{COOH}{\underset{R_1}{HC{-}N{=}HC}}{-}\langle N \rangle}$$

$$\mathrm{\overset{COOH}{\underset{R_2}{HC{-}NH_2}} + OHC{-}\langle N \rangle \; \underset{+H_2O}{\overset{-H_2O}{\rightleftharpoons}} \; \overset{COOH}{\underset{R_2}{HC{-}N{=}HC}}{-}\langle N \rangle}$$

$$\mathrm{\overset{COOH}{\underset{R_2}{C{=}O}} + H_2NH_2C{-}\langle N \rangle \; \underset{+H_2O}{\overset{-H_2O}{\rightleftharpoons}} \; \overset{COOH}{\underset{R_2}{C{=}N{-}H_2C}}{-}\langle N \rangle}$$

Early experimental evidence of a relationship between vitamin B_6 and

378 ... 167, 556 (1951)

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381 E E Snell, *J. Am Chem Soc* ...

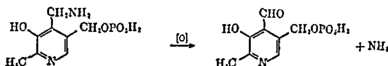
transamination was the finding by Schlenk and Snell[382] in 1945 that the tissues of vitamin B_6-deficient rats exhibited a low order of glutamic-aspartic transaminase activity This finding was confirmed in rats by Ames *et al.*[383] in 1947. Later the glutamic-aspartic system of vitamin B_6-deficient hamsters[384] and the glutamic-pyruvic system (but not the glutamine-keto acid system) of vitamin B_6-deficient rats[385] were found to be reduced in activity Lichstein *et al*[386] in 1945 found that *Streptococcus faecalis* R cells, grown on vitamin B_6-deficient media, exhibited a markedly reduced transaminase activity, which was greatly increased on addition of pyridoxal phosphate. In the studies with vitamin B_6-deficient rats it was found that addition to the tissue extracts of pyridoxal and adenosine triphosphate resulted in an increase in transaminase activity

In 1943 Bellamy and Gunsalus[387, 388, 389] had observed a relationship between the activity of the tyrosine decarboxylase of *S faecalis* and the concentration of pyridoxine in the medium *S faecalis* cells grown on a medium deficient in vitamin B_6 exhibited little or no decarboxylase activity, whereas the addition of pyridoxal restored activity Using dried cells, these workers found that activity was restored by adenosine triphosphate plus pyridoxal, or a crude synthetic pyridoxal phosphate preparation Incubation of the cells (at pH 7 0) with pyridoxamine and pyruvate also led to "codecarboxylase" formation as indicated by restoration of tyrosine decarboxylase activity [390]

Although it appears that pyridoxal phosphate is the prosthetic group for the glutamic-aspartic and glutamic-alanine transaminases as well as for the amino acid decarboxylases, the mechanism of the transamination reaction remains unsettled Of particular interest is the observation of Umbreit *et al*,[391] that, although impure preparations of pyridoxamine phosphate and pyridoxal phosphate activated crude preparations of apotransaminase, only pyridoxal phosphate was active with a highly purified pig heart transaminase Recently activation of the purified pig heart glutamic-aspartic apotransaminase by crystalline pyridoxamine phosphate was reported It

[382] F Schlenk and E E Snell, *J Biol Chem* **157**, 425 (1945)
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[384] G Shwartzman and H Hift, *J Nutrition* **10**, 575 (1951)
[385] A Meister, H P Morris, and S V Tice, *Proc Soc Exptl Biol Med*, **82**, 301 (1953)
[386] H C Lichstein, I C Gunsalus, and W W Umbreit, *J Biol Chem* **161**, 311 (1945)
[387] W D Bellamy and I C Gunsalus, *J Bact* **46**, 573 (1943); **48**, 191 (1944), **50**, 95 (1945)
[388] I C Gunsalus and W D Bellamy, *J Bact* **47**, 413 (1944)
[389] I C Gunsalus and W D Bellamy, *J Biol Chem* **155**, 35, 557 (1944).
[390] I C Gunsalus, W D Bellamy, and W W Umbreit, *J Biol Chem* **155**, 685 (1944)
[391] W W Umbreit, O S O'Kane, and I C Gunsalus, *J Biol Chem* **176**, 629 (1948).

would appear that enzyme-pyridoxamine phosphate combination occurs more slowly (under the experimental conditions employed) than enzyme-pyridoxal phosphate combination.[391a] Although the role of pyridoxamine phosphate in *transamination remains to be completely elucidated, the occurrence* of this compound in natural material[392, 393] and its effect in *in vitro* systems suggest a biological function. It is possible that enzymatic oxidation of pyridoxamine phosphate to pyridoxal phosphate and ammonia occurs.



The conversion of pyridoxamine phosphate to pyridoxal phosphate by enzymatic transamination was recently demonstrated by Meister *et al*[391a] The evidence suggests that a wide variety of α-keto acids are capable of transaminating with pyridoxamine phosphate.

The problem of the structure of pyridoxal phosphate has received much attention It now appears certain that the phosphate group is not attached in the 3 position.[394] It is unquestionably attached in the 5 position.[395, 396, 397] Until recently neither pyridoxamine phosphate nor pyridoxal phosphate had been available in pure form The preparation by Peterson *et al*[393] of *pure crystalline pyridoxamine-5-phosphate, which may be converted to*

' the preparation of pure calcium

[399] may be expected to give im-

oncluded that there is as yet no unequivocal demonstration of the mechanism of amino group transfer Pure characterized substrates and coenzymes, as well as purified enzymes are the minimal requirements necessary for final elucidation of this problem

Although early studies were interpreted to indicate that the role of transamination was relatively limited, present evidence suggests that this reaction is probably one of the most important in the intermediary metabolism of amino acids It is notable that transaminase activity is widely

391a A Meister, H A Sober, and E A Peterson, *J Am Chem Soc.* **74**, 2385 (1952)
392 J. C Rabinowitz and E E Snell, *J Biol Chem* **169**, 643 (1947)
393 W S McNutt and E E Snell, *J Biol Chem.* **173**, 801 (1948).
394 W W. Umbreit and I C Gunsalus, *J Biol Chem* **179**, 279 (1949).
395 D Heyl, E Luz, S A Harris, and K Folkers, *J Am Chem Soc* **73**, 3430 (1951)
396 [illegible] *Soc* **73**, 3437 (1951)
397 c **1952**, 2583
398 , *J. Am Chem Soc* **74**, 570 (1952);
399 *Helv Chim Acta* **34**, 1834 (1951)

distributed in the tissues of the higher animals and plants (cf ref 400), and in microorganisms (cf ref 401) The rapid exchange of amino groups between certain dietary amino acids, and those of the body proteins (Section IV 1) may be explained in terms of transamination By coupled action between glutamic dehydrogenase and transaminase, a mechanism may readily be formulated for the reversible introduction of ammonia into the α-amino groups of most of the amino acids It is also obvious that these two enzyme systems catalyze reactions which link the intermediary metabolism of amino acids with the Krebs cycle Thus pyruvate, oxaloacetate, and α-ketoglutarate represent substrates which may either accept an amino group and ultimately be incorporated in protein, or alternatively be oxidized to carbon dioxide and water

The possibility that transamination may be involved in peptide synthesis (cf ref 402) has been considered, and further study of this subject would be desirable

6 Reductive Deamination

A deamination mechanism which occurs in certain bacteria involves the addition of hydrogen to yield the corresponding saturated fatty acid:

$$\underset{\displaystyle NH_2}{RCHCOOH} + 2H \rightarrow RCH_2COOH + NH_3$$

In certain anaerobic organisms mutual oxidation-reduction between two amino acids may lead to deamination of both amino acids:

$$\underset{\displaystyle NH_2}{R_1CHCOOH} + \underset{\displaystyle NH_2}{R_2CHCOOH} + H_2O \rightarrow R_1CH_2COOH + \underset{\displaystyle O}{R_2\overset{}{C}COOH} + 2NH_3$$

This type of deamination was first observed by Stickland[403] and later studied by others[404, 405, 406] Amino acids found to serve as hydrogen donors include valine, phenylalanine, cysteine, aspartic acid, glutamic acid, alanine, histidine, leucine, and serine, while glycine, ornithine, proline, tryptophan, hydroxyproline, and arginine act as acceptors The "Stickland" reaction between alanine and proline yields δ-aminovaleric acid, pyruvate, and only

400 M J K Leonard and R H Burris, *J Biol Chem* **170**, 701 (1947)
401 H C Lichstein and P P Cohen, *J Biol Chem* **157**, 85 (1945)
402 R M Herbst and D Shemin, *J Biol Chem* **147**, 541 (1943)
403 L H Stickland, *Biochem J* **28**, 1746 (1934), **29**, 889 (1935)
404 D D Woods and A R Trim, *Biochem J* **30**, 1934 (1936)
405 J C Hoogerheide and W Kocholaty, *Biochem J* **32**, 949 (1938)
406 C E Clifton, *J Bact* **39**, 485 (1940)

would appear that enzyme-pyridoxamine phosphate combination occurs more slowly (under the experimental conditions employed) than enzyme-pyridoxal phosphate combination.[391a] Although the role of pyridoxamine phosphate in transamination remains to be completely elucidated, the occurrence of this compound in natural material[392, 393] and its effect in *in vitro* systems suggest a biological function. It is possible that enzymatic oxidation of pyridoxamine phosphate to pyridoxal phosphate and ammonia occurs

$$\text{HO},\ \text{H}_3\text{C},\ \text{CH}_2\text{NH}_2,\ \text{CH}_2\text{OPO}_3\text{H}_2 \text{ (pyridine ring)} \xrightarrow{(O)} \text{HO},\ \text{H}_3\text{C},\ \text{CHO},\ \text{CH}_2\text{OPO}_3\text{H}_2 \text{ (pyridine ring)} + NH_3$$

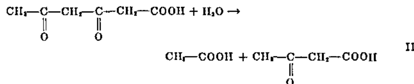
The conversion of pyridoxamine phosphate to pyridoxal phosphate by enzymatic transamination was recently demonstrated by Meister *et al*[326] The evidence suggests that a wide variety of α-keto acids are capable of transaminating with pyridoxamine phosphate.

The problem of the structure of pyridoxal phosphate has received much attention It now appears certain that the phosphate group is not attached in the 3 position.[394] It is unquestionably attached in the 5 position [395, 396, 397] Until recently neither pyridoxamine phosphate nor pyridoxal phosphate had been available in pure form The preparation by Peterson *et al* [398] of pure crystalline pyridoxamine-5-phosphate, which may be converted to pure crystalline pyridoxal phosphate and the preparation of pure calcium pyridoxal-5-phosphate by Viscontini *et al* [399] may be expected to give impetus to studies in this field It must be concluded that there is as yet no unequivocal demonstration of the mechanism of amino group transfer Pure characterized substrates and coenzymes, as well as purified enzymes are the minimal requirements necessary for final elucidation of this problem

Although early studies were interpreted to indicate that the role of transamination was relatively limited, present evidence suggests that this reaction is probably one of the most important in the intermediary metabolism of amino acids It is notable that transaminase activity is widely

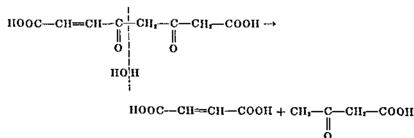
391a [illegible] *Am Chem Soc* 74 2385 (1952)
392 [illegible]
393 [illegible]
394 [illegible]
395 [illegible] 3430 (1951)
396 [illegible]
397 [illegible] Mathias *J Chem Soc* 1952, 2583
398 [illegible] *Am Chem Soc* 74, 570 (1952); [illegible]
399 M Viscontini, [illegible] *Chim Acta* 34, 1834 (1951)

Subsequently it was found that these preparations catalyzed the breakdown of β,δ-diketohexanoic acid to acetate and acetoacetate[408, 409]

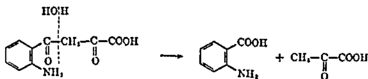


It seems probable that both reactions are catalyzed by the same system. Reaction I has been reported to occur considerably more rapidly than reaction II.

The possibility that the diketo acid hydrolyzing system might play a role in fatty acid catabolism was considered. The studies of Ravdin and Crandall[410] have recently shown that this system hydrolyzes fumarylacetoacetic acid formed by oxidation of homogentisic acid to fumaric and acetoacetic acids (Section V.13):



It is possible that this enzyme system may also be involved in the conversion of kynurenine to anthranilic acid. In this case the postulated intermediate, o-aminobenzoylpyruvic acid, would be hydrolyzed (Section V.12).



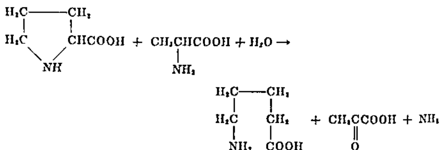
[illegible]

408 [illegible]

409 [illegible]

410 [illegible]

1 mole of ammonia:

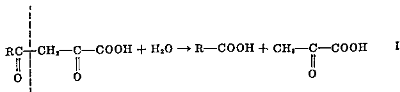


This type of deamination has not been observed in animal tissues or higher plants

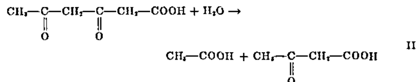
7 Keto and Diketo Acids in Amino Acid Metabolism

which specific L-amino acids were replaced by their keto analogues or, in certain cases, by the corresponding D isomers (Section III.1). Most of the α-keto acids corresponding to the natural amino acids have been prepared, the majority of these by chemical synthesis. Crude uncharacterized keto acid preparations have also been obtained by enzymatic oxidation of the corresponding amino acids Recently, the preparation of a number of pure α-keto acids by enzymatic methods has been described[310] In general, the enzymatic preparation of α-keto acids is less time-consuming and leads to better yields and frequently to a purer product than the available synthetic procedures. Furthermore, certain keto acids, e g, the keto analogues of citrulline, ϵ-N-chloroacetyllysine, asparagine, which can readily be prepared enzymatically, have not yet been prepared by synthesis The preparation of the optically active keto analogues of isoleucine employing D-amino acid oxidase illustrates a special advantage of the enzymatic technique, i e, preservation of stereochemical configuration.[307]

Several years ago,[407] an enzyme was discovered in the liver and kidney of a number of species which catalyzed the hydrolytic splitting of acylpyruvic acids (α,γ-diketo acids) to pyruvic acid and the corresponding fatty acid:

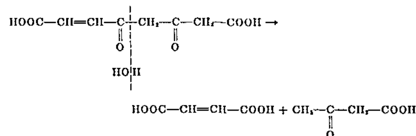


Subsequently it was found that these preparations catalyzed the breakdown of β,δ-diketohexanoic acid to acetate and acetoacetate:[408, 409]

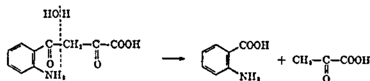


It seems probable that both reactions are catalyzed by the same system Reaction I has been reported to occur considerably more rapidly than reaction II

The possibility that the diketo acid hydrolyzing system might play a role in fatty acid catabolism was considered The studies of Ravdin and Crandall[410] have recently shown that this system hydrolyzes fumarylacetoacetic acid formed by oxidation of homogentisic acid to fumaric and acetoacetic acids (Section V 13):



It is possible that this enzyme system may also be involved in the conversion of kynurenine to anthranilic acid In this case the postulated intermediate, *o*-aminobenzoylpyruvic acid, would be hydrolyzed (Section V 12).



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[407] A Meister and J P Greenstein, *J Biol Chem* 175, 573 (1948).
[408] A Meister, *J Biol Chem* 178, 577 (1949)
[409] R F Witter and E. Stotz, *J Biol Chem* 176, 501 (1948)
[410] R G Ravdin and D I. Crandall, *J Biol Chem.* 189, 137 (1951).

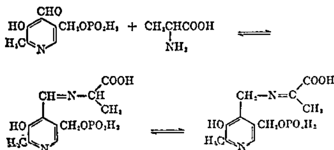
V. Metabolism of the Individual Amino Acids[410a]

1. Alanine

a. β-Alanine. The formation of β-alanine by decarboxylation of aspartic acid has been described in Section IV.4 Although β-alanine arises in certain bacteria by this mechanism, Schenck[61] found no evidence for conversion of aspartic acid to β-alanine, or of aspartylhistidine to carnosine, in the rat Growing rats can synthesize this amino acid,[411] possibly by another mechanism There is no evidence that β-alanine occurs in proteins, although it occurs free to an appreciable extent in muscle and liver.[412] Enzyme systems capable of deaminating β-alanine have been observed in mammalian tissues,[413] and rapid degradation of N^{15}-labeled β-alanine in the intact rat has been observed [414] The synthesis of carnosine from histidine and β-alanine has been demonstrated in liver slices [414a]

b. L-Alanine. The metabolism of this amino acid has been discussed in Section IV. Deamination to pyruvic acid probably represents the initial step in its metabolism.

c. D-Alanine. It is known that a number of bacteria can utilize both the D and L isomers of certain amino acids for growth, whereas others grow only on the L forms An unusual finding is that *Streptococcus faecalis* can utilize D-alanine but not L-alanine in the absence of vitamin B_6 [415, 416] Although D-alanine replaces vitamin B_6 in supporting the growth of this organism, it is not a precursor of vitamin B_6 [101, 102, 417] Recently[418] an enzyme system has been demonstrated in *S. faecalis* which converts both D and L-alanine to the racemic amino acid. The system, tentatively designated "alanine racemase," is apparently specific for alanine and requires pyridoxal phosphate for full activity The mechanism of racemization may be pictured as follows



[410a] An outline of the metabolic pathways of the amino acids is given in Table 7, p 187.

[411] J R Schenck and V du Vigneaud, *J Biol Chem* **153**, 501 (1944)

[412] J R Schenck, *Proc. Soc Exptl Biol Med* **54**, 6 (1943)

[413] B Kisch, *Enzymologia* **1**, 97 (1936)

[414] H. D Hoberman and J Graff, *J Biol Chem* **186**, 373 (1950).

By such a mechanism, the reversible formation of a Schiff's base complex between alanine and pyridoxal phosphate leads to destruction of the asymmetric center and thus produces racemization It was recently found in the writer's laboratory that non-enzymatic racemization of L-alanine occurred when this amino acid and pyridoxal were heated for short periods [419]

2 Glycine, Sarcosine, Serine, and Threonine

The oxidation of glycine to glyoxylic acid by liver and kidney preparations was described by Ratner, Nocito, and Green [420] The enzyme responsible for the oxidation was designated "glycine oxidase," and evidence was presented indicating that it was different from D-amino acid oxidase Inactivated glycine oxidase was stimulated by flavin adenine dinucleotide Sarcosine is deaminated by this preparation, yielding glyoxylic acid and methylamine Handler *et al* [421] described a system in liver which demethylates sarcosine to formaldehyde and glycine The conversion of sarcosine to glycine has also been demonstrated in intact animals [422, 423] In a recent study by Horner and Mackenzie[424] rats were fed a diet containing nonisotopic sarcosine, and betaine or methionine labeled with C^{14} in the methyl group The methyl group of the isolated urinary sarcosine was found to be labeled, indicating that sarcosine is a metabolite in the rat Weinhouse and Friedmann[425] found that glycolic and glyoxylic acids, when injected intraperitoneally in rats, were converted to glycine Thus biological mechanisms exist for the deamination and reamination of glycine

An important advance in the understanding of the metabolism of glycine was the demonstration that glycine and serine were interconvertible Shemin[426] first showed the conversion of serine to glycine, and the conversion of glycine to serine was demonstrated by Goldsworthy *et al*,[427] Winnick

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414a H M Williams and W A Krehl, *J Biol Chem* **196**, 443 (1952)

415 E E Snell and B M Guirard, *Proc Natl Acad Sci U S* **29**, 66 (1943).

416 E E Snell, *J Biol Chem* **158**, 497 (1945)

417 W D Bellamy and I C Gunsalus, *J Bact* **50**, 95 (1945)

418 W A Wood and I C Gunsalus, *J Biol Chem* **190**, 403 (1951)

419 Unpublished data, *cf* J Olivard, D E Metzler, and E E Snell, *J Biol Chem* **190**, 669 (1952)

420 S Ratner, V Nocito, and D E Green, *J Biol Chem* **152**, 119 (1944)

421 P Handler, F Bernheim, and J R Klein, *J Biol Chem* **138**, 211 (1941)

422 L D Abbott and H B Lewis, *J Biol Chem* **131**, 479 (1939), **137**, 535 (1941).

423 K Block and R Schoenheimer, *J Biol Chem* **135**, 99 (1940)

424 W H Horner and C A Mackenzie, *J Biol Chem* **187**, 15 (1950)

425 S Weinhouse and B Friedmann, *J Biol Chem* **191**, 707 (1951)

426 D Shemin *J Biol Chem* **162**, 297 (1946)

427 P D Goldsworthy, T Winnick, and D M Greenberg, *J Biol Chem* **180**, 341 (1949)

et al.,[428] and Siekevitz *et al.*[429, 430] Sakami[431] demonstrated that formate condenses with glycine to form serine. His data indicate that the formate carbon becomes the β-carbon of serine:

$$CH_2NH_2C^{14}OOH + HC^{14}OOH \rightarrow C^{14}H_2OHCHNH_2C^{14}OOH$$

According to Siekevitz and Greenberg[430] in studies with liver slices, the α-carbon atom of glycine can form formate which may condense with another molecule of glycine to yield serine. It is of interest that the α-carbon atom of glycine and formate are precursors of carbon atoms 2 and 8 of uric acid in the pigeon.[432] The mechanism of formate formation from glycine probably involves oxidative decarboxylation of glyoxalate.[433] Other sources of formate include labile methyl groups and carbon 2 of the imidazole ring of histidine.

Levine and Tarver[434] found that the administration of serine labeled with C^{14} in the β position to rats led to the appearance of β-labeled ethanolamine in the lipide fraction of the liver, suggesting that the pathway from glycine to ethanolamine is via serine. Similar conclusions were reached by Arnstein.[435] Elwyn and Sprinson[436] reported that the β-carbon of L-serine was utilized in the pigeon for the ureide carbon atoms of uric acid to about the same extent as was the nitrogen for position 7.[436] These workers have also found a reduced conversion of serine to glycine in folic acid-deficient rats. Other evidence suggests that folic acid is involved in the incorporation of formate into serine.[437]

The glycine carbon atoms also appear in purines,[438] hemin,[439] creatine,[440] and uric acid.[441, 442] In *Diplococcus glycinophilus*, glycine is anaerobically converted to acetic acid probably by condensation of 2 molecules of glycine

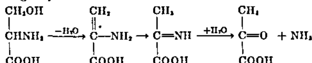
[428] T Winnick, I Moring-Claesson, and D M Greenberg, *J. Biol. Chem* **175**, 127 (1948)
[429] P Siekevitz, T Winnick, and D M Greenberg, *Federation Proc.* **8**, 250 (1949)
[430] P Siekevitz and D M Greenberg, *J. Biol Chem* **180**, 845 (1949).
[431] W Sakami, *J Biol Chem* **176**, 995 (1948)
[432] J L Karlsson and H A Barker, *J Biol Chem* **177**, 597 (1949)
[433] B Friedmann, H I Nakada, and S Weinhouse, *Federation Proc* **10**, 185 (1951)
[434] M Levine and H Tarver, *J Biol Chem* **184**, 427 (1950)
[435] H R V Arnstein, *Biochem J* **48**, 27 (1951)
[436] D Elwyn and D Sprinson, *J Biol Chem* **184**, 465, 475 (1950)
[437] [illegible] 705 (1950)
[438] [illegible]
[439] [illegible]
[440] K Bloch and R Schoenheimer, *J Biol Chem* **138**, 167 (1941).
[441] J. C Sonne, J M Buchanan, and A M Delluva, *J Biol. Chem* **173**, 69 (1948)
[442] J. M Buchanan, J. C Sonne, and A M Delluva, *J Biol Chem* **173**, 81 (1948)

and subsequent decarboxylation of the terminal carbon atoms[443] A similar condensation reaction may occur in the rat, leading to aspartate synthesis[444]

Paretsky and Werkman[445] have found that a strain of *Achromobacter* oxidizes glycine with the formation of ammonia and hydrogen peroxide. Formaldehyde, probably formed from glyoxylic acid, was considered to be an intermediate in this reaction

in the body (Section IV 1) The synthesis of hippuric acid takes place in the liver

In certain bacteria, DL-serine is deaminated to pyruvate, and DL-threonine to α-ketobutyrate[446] The reaction with serine presumably involves the following steps:



In rat kidney slices hydroxypyruvic acid was found to be the deamination produce of D-serine[447] There is now evidence for the existence of both a D-serine deaminase and an L-serine deaminase in *E coli* The former is activated by pyridoxal phosphate[447a], and the latter by adenylic acid and glutathione.[447b]

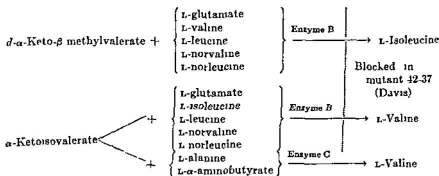
3 Isoleucine and Valine

The discovery of a *Neurospora* mutant which required valine and isoleucine[448] initiated a series of studies on the synthesis of these amino acids by this organism and *E coli* Early studies demonstrated an inhibition of valine synthesis by an isoleucine precursor, originally considered to be the corresponding α-keto acid[449] An *E coli* mutant requiring isoleucine was found to accumulate α-keto-β-methylvaleric and α-ketoisovaleric acids[450] The original studies on *Neurospora*[449] were complicated by the use of unpurified keto acids and a synthetic preparation considered to be α-keto-β-

[443] H A Barker, B E Volcani, and B P Cardon, *J Biol Chem* **173**, 803 (1948)
[444] D B Sprinson, *J Biol Chem* **178**, 529 (1949)
[445] D Paretsky and C H Werkman, *Arch Biochem* **25**, 288 (1950)
[446] E Chargaff and D B Sprinson, *J Biol Chem* **151**, 273 (1943)
[447] D B Sprinson and E Chargaff, *J. Biol Chem* **164**, 411 (1946)
[447a] D E Metzler and E E Snell, *J Biol Chem* **198**, 363 (1952)
[447b] W A Wood and I C Gunsalus, *J Biol. Chem* **181**, 171 (1949).
[448] D Bonner, E L Tatum, and G W Beadle, *Arch Biochem* **3**, 71 (1943).
[449] D Bonner, *J Biol Chem* **166**, 545 (1946)
[449a] H E Umbarger and B Magasanik, *J. Am Chem Soc.* **74**, 4256 (1952)
[450] H. E Umbarger and J H Mueller, *J Biol Chem* **189**, 277, 287 (1950)

methylvaleric acid but subsequently found to be α-ketobutyric acid.[451] An isoleucine precursor, considered to be α-β-dihydroxy-β-ethylbutyric acid, has been isolated. This compound, and an analogous valine intermediate, were isolated from *Neurospora* culture filtrates.[452, 453] Studies with radioactive acetate led to the conclusion that the 4-carbon straight chains of isoleucine and valine are formed, at least in part, by "head-to-tail" coupling of 2 acetate molecules, that the ethyl group of isoleucine arises from acetate, and that the methyl group of acetate contributes to the methyl group of valine.[454] In a recent reinterpretation of these studies[451] a metabolic scheme is presented in which the α-β-dihydroxy acids are converted to their respective α-keto acids, which are aminated to the corresponding amino acids. Certain isoleucine requiring mutants can grow on D-threonine, L-threonine, α-aminobutyric acid, and α-ketobutyric acid, suggesting other possible metabolic relationships.[450]

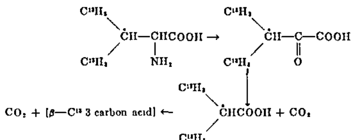
In the studies on *Neurospora* and *E. coli*, mutants possessing an absolute requirement for isoleucine and a relative requirement for valine were frequently obtained. It was suggested that the requirement for valine was due to inhibition of the amination of α-ketoisovalerate by accumulation of α-keto-β-methylvalerate.[445, 449, 449a] Recent work indicates that the valine requirement can be supplied by equivalent concentrations of L-alanine or L-α-aminobutyrate.[874] This mutant is therefore characterized by an absolute requirement for L-isoleucine and a relative requirement for L-valine, L-alanine, or L-α-aminobutyrate. The genetic block in this mutant is associated with loss of a specific transaminase (enzyme B), which is the sole system for catalysis of transamination involving isoleucine, and a major (but not the sole) system for valine transamination. The growth requirement for valine is not absolute, since some α-ketoisovalerate is aminated by reactions between this keto acid and alanine or α-aminobutyric acid catalyzed by "enzyme C." These alternative pathways of valine biosynthesis in *E. coli* may be represented as follows:



[451] H. E. Umbarger and E. A. Adelberg, *J. Biol. Chem.* **192**, 883 (1951).

It has been found that at least one microorganism is capable of inverting the stereochemical configuration of the β-carbon atom of isoleucine. Thus *L. arabinosus* can convert *l*-α-keto-β-methylvalerate and L-alloisoleucine to L-isoleucine[377] A study of the optical configuration of the dihydroxy acid precursors of isoleucine would be of interest.

The administration of valine to phlorizinized dogs[375] and fasted rats[376] resulted in the formation of carbohydrate The glyconeogenic effect of L-valine in rats was investigated with labeled valine by Fones *et al.*[455] On the basis of these studies, the following metabolic pathway appears plausible



The relatively high concentrations of isotope found in the 1,6 and 2,5 positions of the isolated glucose (from liver glycogen) suggested that valine was metabolized to a 3-carbon acid which was utilized for glycogen formation[456]

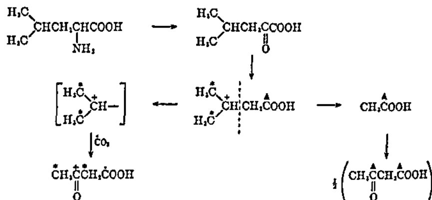
4 Leucine

Early studies indicated that the metabolism of leucine in animals gave rise to ketone bodies Embden *et al*[457] had noted that isovaleric acid, which they postulated as a breakdown product of leucine, produced ketone bodies in perfusion experiments Similar conclusions were possible from studies on intact animals[458] Bloch[459] observed the conversion of deuterium-labeled isovaleric acid and leucine to 2-carbon fragments Coon and Gurin,[460]

[452] E A Adelberg, D M Bonner, and E L Tatum, *J Biol Chem* **190**, 837 (1951)
[453] E A Adelberg, *J Bact* **61**, 365 (1951)
[454] E L Tatum and E A Adelberg, *J Biol Chem* **190**, 843 (1951)
[455] W S Fones, T P Waalkes, and J White, *Arch Biochem Biophys* **32**, 89 (1951).
[456] E A Peterson, W C Fones, and J White, *Arch Biochem Biophys* **36**, 323 (1952)
[457] G Embden, H Salomon, and F Schmidt, *Beitr. Chem Physiol u. Path* **8**, 129 (1906)
[458] A I Ringer E M Frankel, and L Jonas, *J Biol Chem* **14**, 525 (1913).
[459] K Bloch, *J Biol Chem* **155**, 255 (1944)
[460] M J Coon and S Gurin, *J Biol Chem* **180**, 1159 (1949)

Coon,[461] and Zabin and Bloch[462] have studied the degradation of leucine to acetoacetate by liver slices with isotopic techniques

Carbons 1 and 2 of isovaleric acid (or the α- and β-carbons of leucine) gave rise to 2-carbon fragments which were believed to condense to form acetoacetic acid. The carbons of the methyl groups of the isopropyl residue were incorporated into the methyl and methylene groups of acetoacetic acid, while, with 3-labeled isovaleric acid or 4-labeled leucine, isotope was found primarily in the carbonyl position of acetoacetic acid The fixation of isotopic carbon dioxide in the carboxyl group of acetoacetate was demonstrated The following metabolic scheme was proposed by Coon:[461]



Sprinson and Rittenberg[463] fed rats leucine labeled with deuterium in the α, β, and γ positions and N^{15} in the amino group, and leucine was subsequently isolated from the tissue proteins Since the dilution of the β- and γ-deuterium atoms was similar, they concluded that α-β dehydrogenation of this amino acid did not occur Although the β- and γ-deuterium atoms accompany their carbon atoms during reversible deamination, the data do not eliminate the possibility of the occurrence of α-β unsaturation during a non-reversible degradative process It is also conceivable that an enzyme-directed reversible α-β unsaturation occurs, which would preserve the original labeling

5 Glutamic Acid

The significance of glutamic acid in transamination and its decarboxylation to γ-aminobutyric acid have been discussed above (Sections IV.4 and IV.5) The reversible conversion of glutamate to α-ketoglutarate represents an important link with the Krebs cycle It has recently been shown that

461 M J Coon, *J. Biol. Chem* **187**, 71 (1950)
462 I Zabin and K. Bloch, *J. Biol Chem* **185**, 117 (1950)
463 D. B Sprinson and D Rittenberg, *J. Biol Chem.* **184**, 405 (1950).

interruption of the Krebs cycle by ammonia is due to the amination of α-ketoglutarate [464]

The reversible amination of α-ketoglutarate by ammonia is catalyzed by glutamic dehydrogenase, an enzyme of considerable activity and widespread occurrence This system has been studied in microorganisms,[465, 466, 467] higher plants,[468, 469] and animal tissues [470, 471] Although the reaction is fundamentally the same as that catalyzed by the general amino acid oxidases, the enzyme utilizes diphosphopyridine nucleotide (DPN) or triphosphopyridine nucleotide (TPN) as coenzymes [472] The glutamic dehydrogenase of liver can function with either DPN and TPN, while the system found in certain microorganisms utilizes TPN only In glutamate synthesis, which requires hydrogen, the system is probably coupled with other dehydrogenase systems, such as the malic, β-hydroxybutyric, and isocitric dehydrogenases It may also be coupled with the decarboxylation of α-ketoglutarate to succinate Glutamic dehydrogenase has recently been purified from liver and crystallized [473, 474] A glutamic acid racemase has been discovered in *L arabinosus* [474a, 474b] The system catalyzes the interconversion of the D and L isomers of glutamic acid and appears to require pyridoxal phosphate (see Section V 1 c)

A number of studies have suggested that glutamic acid may play a significant role in the metabolism of nerve tissue [475] The conversion of D-glutamic acid to pyrrolidone carboxylic acid in the rat was reported by Ratner [476] The relationships between glutamic acid, ornithine, arginine, and proline are discussed in Section V 9

A connection between the metabolism of glutamic acid and folic acid is suggested by recent studies in which rats fed a purified diet containing

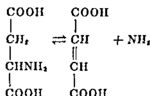
[464] R O Recknagel and V R Potter, *J Biol Chem* **191**, 263 (1951)
[465] E Adler, G Gunther, and J E Everett, *Z physiol Chem* **255**, 27 (1938)
[466] H von Euler, E Adler, and T Steenhoff Erickson, *Z physiol Chem* **248**, 227 (1937)
[467] E Adler, V Hellström, G Günther, and H von Euler, *Z physiol Chem*, **255**, 14 (1938)
[468] M Damodaran and K R Nair, *Biochem J* **32**, 1064 (1938)
[469] J Berger and G S Avery, *Jr Am J Botany* **30**, 290, 297 (1943), **31**, 11 (1944)
[470] H von Euler, E Adler, G Günther, and N B Das, *Z physiol Chem* **254**, 61 (1938)
[471] J G Dewan, *Biochem J* **32**, 1378 (1938), **33**, 549 (1939)
[472] A H Mehler, A Kornberg, S Grisolia, and S Ochoa, *J Biol Chem* **174**, 961 (1948)
[473] H J Strecker, *Arch Biochem Biophys* **32**, 448 (1951)
[474] J A Olson and C B Anfinson, *J Biol, Chem* **197**, 67 (1952)
[474a] S A Narrod and W A Wood, *Arch Biochem Biophys* **35**, 462 (1952)
[474b] P Ayengar and E Roberts, *J Biol Chem* **197**, 453 (1952)
[475] H Waelsch, *Advances in Protein Chem* **6**, 301 (1951)
[476] S Ratner, *J Biol Chem* **152**, 559 (1944)

succinylsulfathiazole were found to excrete five to ten times as much glutamic acid in the urine as did control animals.[477] The increased glutamate excretion was reversed by folic acid administration

6. Aspartic Acid

The role of aspartic acid in transamination reactions and its enzymatic decarboxylation to α-alanine and β-alanine has been discussed above (Sections IV.4 and IV.5). It is probable that the major pathway for the synthesis and degradation of aspartic acid in higher animals proceeds via transamination (cf ref 478) The reversible formation of oxaloacetic acid from aspartic acid indicates a direct relationship with carbohydrate metabolism

In certain microorganisms[479-483] and higher plants[481] aspartate is reversibly deaminated to fumarate by the enzyme aspartase:



There is evidence that two aspartases may exist and that adenylic acid functions as a coenzyme in one of these systems [483] The fumarate formed may be reduced to succinate or converted to malate.

The general D- and L-amino acid oxidases have a relatively low activity toward the respective isomers of aspartic acid Recently, however, a D-aspartic acid oxidase was described in pig kidney by Still *et al* [484] The enzyme has been purified and shown to possess flavin adenine dinucleotide as the prosthetic group [485] The enzyme preparation also has some activity toward D-glutamic acid

In certain microorganisms the synthesis of aspartic acid requires biotin,

[477] H A Bakerman, M Silverman, and F S Daft, *J Biol Chem* **188**, 117 (1951).
[478] H I Nakada and S Weinhouse, *J Biol Chem* **187**, 663 (1950)
[479] J H Quastel and B Woolf, *Biochem J* **20**, 545 (1926)
[480] R P Cook and B Woolf, *Biochem J* **22**, 474 (1928)
[481] A I Virtanen and J Tarnanen, *Biochem Z* **250**, 193 (1932)
[482] B Woolf, *Biochem J* **23**, 472 (1929)
[483] E F Gale, *Biochem. J* **32**, 1583 (1938)
[484] J L Still, M V Buell, W E Knox, and D E Green, *J. Biol Chem* **179**, 831 (1949)
[485] J L Still and E Sperling, *J. Biol Chem* **182**, 585 (1950)

and the requirement for biotin is considerably reduced by addition of aspartic acid [486, 489] Biotin is apparently active for the fixation of carbon dioxide in aspartic acid [490]

7 Glutamine and Asparagine

Glutamine, which is widely distributed in animal tissues, both as free glutamine and in protein, has been implicated in a number of biological mechanisms Thus glutamine plays a role in detoxification,[492] in the metabolism of the central nervous system,[475] and as a precursor of urinary ammonia [492] Glutamine is considered, quite logically, to function in the storage and transport of ammonia Mechanisms involved in the glutamine ⇌ glutamic reaction will be considered below The suggested role of glutamine in urea synthesis[493, 494] does not appear probable on the basis of recent work. On the other hand its possible relationship to the metabolism of nucleotides and nucleosides deserves further investigation In plants the accumulation of glutamine has received considerable attention (for reviews of this subject, see refs 492, 495, 496) There is also evidence that glutamine is an essential growth factor for certain microorganisms.[497-501] Glutamine may be involved in peptide and protein synthesis, although it is apparently not an intermediate in glutathione synthesis in animal tissues [503] The recent studies

[486] S A Koser, M H Wright, and A Dorfman, *Proc Soc Exptl Biol Med* **61**, 204 (1942)

[487] J L Stokes, A Larsen, and M Gunness, *J Bact* **54**, 219 (1947)

[488] H A Lardy, R L Potter, and C A Elvehjem, *J Biol Chem* **169**, 451 (1947)

[489] H Shive and L L Rogers, *J Biol Chem* **169**, 453 (1947)

[490] H A Lardy, R L Potter, and R H Burris, *J Biol Chem* **179**, 721 (1949)

[491] H P Broquist and E E Snell, *J Biol Chem* **188**, 431 (1951)

[492] R M Archibald, *Chem Revs* **37**, 161 (1945)

[493] F Leuthardt, *Z physiol Chem* **252**, 238 (1938), **265**, 1 (1940)

[494] F Leuthardt and B Glasson, *Helv Chim Acta* **25**, 630 (1942), *Helv Physiol et Pharmacol Acta* **1**, 221 (1943)

[495] A C Chibnall, Protein Metabolism in the Plant, Yale University Press, New Haven, Conn , 1939

[496] H L Street, Advances in Enzymol **9**, 39 (1949)

[497] H McIlwain, P Fildes, G P Gladstone, and B J C Knight, *Biochem J* **33**, 223 (1939)

[498] H McIlwain, *Biochem J* **33**, 1942 (1939)

[499] C M Lyman, K A Kuiken, L Blotter, and F Hale, *J Biol Chem* **157**, 395 (1945)

[500] L R Hac F E Snell, and R Williams, *J Biol Chem* **159**, 273 (1945)

[501] M A Pollack and M Lindner, *J Biol Chem* **143**, 655 (1942); **147**, 183 (1943)

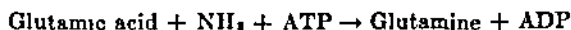
[502] P Ayengar, E Roberts, and G B Ramasarma, *J Biol Chem* **193**, 781 (1951).

[503] R B Johnston and K Bloch, *J Biol Chem* **179**, 493 (1949)

of Waelsch and collaborators[503, 504, 505] on the glutamo- and asparto-transferase systems are of interest in this connection.

The hydrolytic breakdown of glutamine to glutamic acid and ammonia, catalyzed by the enzyme glutaminase, has been found to occur in a variety of animal,[506–509] plant,[510] and bacterial[511, 512] systems. The glutaminase activity of animal tissues is considerably augmented by the presence of certain anions, such as phosphate, arsenate, and sulfate.[509] It should be noted that phosphate buffer was employed in early studies on glutaminase.[506] The dramatic effect of phosphate on rat liver glutaminase activity is such that practically no hydrolysis of glutamine occurs in extracts of this tissue unless phosphate (or certain other anions) are added. It has been suggested that the mechanism of phosphate-activated glutamine deamidation involves formation of a labile phosphate ester of the imino form of the amide.[513] The effect of pyruvate and other α-keto acids on glutamine deamidation[514] involves a transamination mechanism (see Section IV 5). There is evidence for the occurrence of several separate glutaminases in animal tissues,[508] and for the non-identity of glutaminase and asparaginase.[515]

The synthesis of glutamine from glutamate and ammonia does not represent a simple reversal of the glutaminase reaction. Krebs demonstrated the synthesis of glutamine in tissue slices and noted that the reaction required energy. Recent studies with cell-free systems by Speck[516] and Elliott[517] indicate that the reaction requires adenosine triphosphate and magnesium ions. The synthesis may be described as follows:



Glutamic acid + NH_3 + ATP → Glutamine + ADP

The intermediate formation of a γ-glutamyl energy-rich phosphate com-

[503] N Grossowicz, E Wainfan, E Borek, and H Waelsch, *J Biol. Chem.* **187**, 111 (1952)
[504] H Waelsch, P Owades, E Borek, N Grossowicz, and M. Schou, *Arch Biochem* **27**, 237 (1950).
[505] M Schou, N Grossowicz, and H Waelsch, *J. Biol Chem* **192**, 187 (1951)
[506] H A Krebs, *Biochem J* **29**, 1951 (1935)
[507] V E Price and J P Greenstein, *J Natl Cancer Inst* **7**, 275 (1947)
[508] J P Greenstein and F M Leuthardt, *Arch Biochem* **17**, 105 (1948)
[509] J A Shepherd and G Kalnitsky, *J Biol Chem* **192**, 1 (1951)
[510] C E Grover and A C Chibnall, *Biochem J* **21**, 857 (1927).
[511] ... *J Gen Microbiol* **2**, 186 (1948)
[512]
[513] ... 47)
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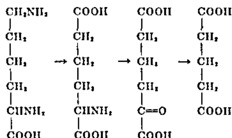
pound has been considered The synthesis is inhibited by methionine sulfoxide,[516, 518] which is known as a bacterial antimetabolite [519]

The accumulation of asparagine in plants is well recognized [492, 495, 496] Asparagine also occurs in animal tissues, although its function is undoubtedly less important than glutamine. Asparaginase activity is widely distributed in nature, having been reported in microorganisms[520, 521, 522] and in plant[510] and animal tissues.[507, 513, 523-529] The synthesis of asparagine may occur by a reaction analogous to the synthesis of glutamine

8 Lysine

The metabolism of lysine (and threonine) in animals differs from that of the other amino acids in that there is apparently no mechanism for reversible amination Administration of $N^{15}H_3$ to rats did not lead to incorporation of isotope in the α-amino group of lysine [530] Furthermore, lysine labeled with deuterium and N^{15} was incorporated without change in the D:N ratio [531] There is also evidence indicating that only slight labilization of the α-hydrogen of lysine occurs in the intact rat [532]

A mechanism for the degradation of lysine in guinea pig liver homogenates was discovered by Borsook *et al* [533] It was observed that ϵ-C^{14}-labeled lysine was converted to α-aminoadipic acid, α-ketoadipic acid, and glutaric acid·



[518] W H Elliott and E F Gale, *Nature* **161**, 129 (1948)

[519] H Waelsch, P Owades, H K Miller, and E Borek, *J Biol Chem* **166**, 273 (1946)

[520] K Schmalfuss and K Mothes, *Biochem Z* **221**, 134 (1930)

[521] S Utzino and M Imaizumi, *Z physiol Chem* **253**, 51 (1938)

[522] G Gorr and J Wagner, *Biochem Z* **254**, 1 (1932)

[523] M Errera and J P Greenstein, *J Natl Cancer Inst* **7**, 437 (1947)

[524] O von Fürth and M Friedmann, *Biochem Z* **26**, 435 (1910)

[525] J P Greenstein and C E Carter, *J Natl Cancer Inst* **7**, 57 (1946)

[526] J M Gonsalves, V E Price, and J P Greenstein, *J Natl Cancer Inst* **7**, 281 (1947)

[527] M E Errera and J P Greenstein, *J Natl Cancer Inst* **7**, 285 (1947).

[528] A Clementi, *Arch intern physiol* **19**, 369 (1922)

The conversion of α-aminoadipic acid to α-ketoadipic acid may take place by transamination[533] Although α-aminoadipic acid cannot substitute for lysine in supporting the growth of rats and certain bacteria,[534] a *Neurospora crassa* mutant can utilize this dicarboxylic acid in place of lysine It has recently been demonstrated that α-aminoadipic acid is a precursor of lysine in *Neurospora crassa*.[535] The utilization of α-amino-ε-hydroxycaproic acid by *Neurospora* mutants led to the suggestion that this lysine analogue may be a precursor of lysine in *Neurospora*.[536] On the other hand, this derivative cannot replace lysine in the diet of rats (Section III.1).

The mechanism elucidated by Borsook *et al.* may not represent the only pathway of lysine degradation It should be noted that the rate of oxidation of lysine observed by these workers was relatively low. Because ε-N-substituted lysine derivatives are more susceptible to enzymatic oxidation than is lysine itself (Section IV.3), it has been suggested that acetylation of the ε-amino group may represent the first step in the degradation of lysine.[537]

9. Arginine, Ornithine, and Citrulline

In 1932, Krebs and Henseleit[273] suggested a mechanism for the formation of urea (Section IV.1) These workers noted the catalytic effect of ornithine and citrulline on urea formation and postulated that mechanisms existed for the synthesis of citrulline from ornithine, and of arginine from citrulline The hydrolysis of arginine by arginase to ornithine and urea completed the cycle

Arginase had been known since 1904,[538] and it was considered probable that urea formation was due mainly to hydrolysis of arginine Arginase activity is widely distributed in mammalian tissues and those of other vertebrates[539] Particularly high concentrations are found in liver[540] and mammary tissue[541] The hydrolysis of arginine is also catalyzed by systems

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529 G Steenholt, *Acta Physiol Scand.* 8, 342 (1944)

530 G L Foster, R Schoenheimer, and D Rittenberg, *J Biol Chem* 127, 319 (1939)

531 N Weissmann and R Schoenheimer, *J Biol Chem* 140, 779 (1941)

532 I Clark and D Rittenberg, *J Biol Chem* 189, 521 (1951)

533 H Borsook, C L Deasy, A J Haagen-Smit, G Keighley, and P H Lowy, *J Biol Chem* 179, 689, 705 (1949)

534 [illegible]

535 [illegible]

536 [illegible]

537 [illegible]

538 [illegible]

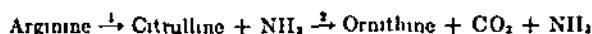
539 [illegible]

540 [illegible]

541 [illegible]

found in certain microorganisms[542] and higher plants [543] The enzyme has been considered specific for L-arginine, although it exhibits some activity toward octopine,[544] α-N-benzoyl-arginine[545] and L-α-uramido-δ-guanidovaleric acid [546] Although δ-N-methylarginine,[547] arginine phosphate,[548] γ-guanidinobutyric acid,[547] and ε-guanidinocaproic acid[549] are not split, the enzyme attacks canavanine[550] and agmatine [551] The hydrolysis of canavanine to urea and canaline was attributed to a separate enzyme,[552] although this has been contested [550] The mechanism and kinetics of arginase activity and its enhancement by certain divalent metal ions (Co^{++}, Ni^{++}, Fe^{++}, Mn^{++}) has received considerable attention [553, 554, 555]

The degradation of arginine in certain bacteria involves the following reactions [555a, 555b]



$$\text{Arginine} \xrightarrow{1} \text{Citrulline} + NH_3 \xrightarrow{2} \text{Ornithine} + CO_2 + NH_3$$

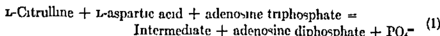
Reaction 1 is catalyzed by arginine desimidase,[555b, 555c] which has been separated from the system responsible for citrulline degradation This degradative pathway is somewhat similar to the synthetic route in animal tissues

Although the Krebs-Henseleit cycle is probably a correct representation of the general outline of urea formation, the intimate mechanisms involved have not been completely elucidated Suggestions that other mechanisms may exist for the formation of urea are not as yet supported by conclusive evidence

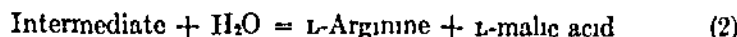
Borsook and Dubnoff[556] and later Krebs[557] established that the anaerobic conversion of citrulline to arginine was stimulated by aspartate and glu-

542 S Edlbacher, M Becker, and A V Segesser, *Z physiol Chem* **255**, 53 (1938)
543 C H Stock, M E Perkins, and L Hellerman, *J Biol Chem* **125**, 753 (1938)
544 S Akasi, *J Biochem (Japan)* **26**, 129 (1937)
545 K Felix, H Muller, and K Dirr, *Z physiol Chem* **178**, 192 (1928)
546 A Hunter, *Biochem J* **32**, 826 (1938)
547 K Thomas, J Kapfhammer, and B Flaschenträger, *Z physiol Chem* **124**, 75 (1922)
548 O Meyerhof and K Lohmann, *Biochem Z* **196**, 22 (1928)
549 K Thomas, *Z physiol Chem* **88**, 465 (1913)
550 M Damodaran and K G A Narayanan, *Biochem J* **34**, 1449 (1940)
551 M M Richards and L Hellerman, *J Biol Chem* **134**, 237 (1940)
552 M Kitagawa and T Tomita, *Proc Imp Acad (Tokyo)* **5**, 380 (1929)
553 L Hellerman and M E Perkins, *J Biol Chem* **112**, 175 (1935)
554 S Edlbacher and H Baur, *Z physiol Chem* **254**, 275 (1938)
555 A B Anderson, *Biochem J* **39**, 139 (1945)
555a G C Schmidt, M A Logan, and A A Tytell, *J Biol Chem* **198**, 771 (1952)
555b E L Oginsky and R F Gehrig, *J Biol. Chem* **198**, 791, 799 (1952)
555c F Horn *Z Physiol Chem* **216**, 244 (1933).
556 H Borsook and J W Dubnoff, *J Biol Chem* **141**, 717 (1941)
557 H A Krebs *Biochem J* **36**, 758 (1942)

tamate Ratner[558, 559, 560] has studied a system catalyzing the synthesis of arginine from citrulline. Two enzymatic steps were originally postulated, and later the enzymes responsible were separated:



$$\text{L-Citrulline} + \text{L-aspartic acid} + \text{adenosine triphosphate} = \text{Intermediate} + \text{adenosine diphosphate} + PO_4^{-} \quad (1)$$



$$\text{Intermediate} + H_2O = \text{L-Arginine} + \text{L-malic acid} \quad (2)$$

It appears probable that reaction 1 represents an over-all reaction mechanism

Studies on *Neurospora* mutants[561] suggested that the conversion of ornithine to citrulline involved two steps. The first of these concerned the addition of carbon dioxide to ornithine, and the second, the introduction of ammonia into the molecule. The formation of urea from ornithine with fortified liver homogenates was demonstrated by Borsook and Dubnoff.[562] Under these conditions, these workers observed the accumulation of a substance which was probably citrulline Cohen and collaborators[563-567] have studied this system in detail and have separated the system responsible for citrulline synthesis The synthesis by a soluble system required adenosine triphosphate, magnesium ions, carbon dioxide, ammonia, and carbamylglutamic acid However, studies with carbamylglutamic acid labeled with C^{14} in the carbamyl group, C^{14}-labeled carbon dioxide, and N^{15}-labeled ammonia indicated that neither the carbon nor the nitrogen of the carbamyl group appeared in the carbamyl group of citrulline The function of carbamylglutamic acid in this system remains to be explained. It has been suggested that a phosphate derivative of carbamylglutamic acid is involved. The synthesis of citrulline from ornithine and glutamate was decreased in liver preparations obtained from biotin-deficient rats, while the synthesis from ornithine and carbamylglutamic acid proceeded at equivalent rates in deficient and control preparations [568, 569] Hirs and Rittenberg[570] have concluded that the α- and δ-amino groups of ornithine contribute to a negligible

558 S Ratner and A Pappas, *J Biol Chem* **179**, 1183, 1199 (1949).
559 S Ratner, *Federation Proc* **8**, 603 (1949)
560 S Ratner and B Petrack, *J Biol Chem* **191**, 693 (1951)
561 A M. Srb and N H Horowitz, *J Biol Chem* **154**, 129 (1944).
562 H. Borsook and J W Dubnoff, *J Biol Chem* **169**, 461 (1947)
563 P P Cohen and M Hayano, *J Biol. Chem* **166**, 239, 251 (1946), **170**, 687 (1947), **172**, 405 (1948)
564 P. P Cohen and S Grisolia, *J Biol Chem* **174**, 389 (1948), **182**, 747 (1950)
565 S Grisolia, S B Koritz, and P P Cohen, *J Biol Chem* **191**, 181 (1951)
566 S Grisolia and P P. Cohen, *J Biol Chem* **191**, 189 (1951)
567 S Grisolia, R. H Burris, and P P Cohen, *J. Biol Chem* **191**, 203 (1951)
568 P. R MacLeod, S Grisolia, P P. Cohen, and H A Lardy, *J Biol Chem* **180**, 1003 (1949)
569 G. Feldott and H A Lardy, *J Biol Chem* **192**, 447 (1951)
570 C. H. W. Hirs and D Rittenberg, *J Biol Chem* **186**, 429 (1950)

extent to urea formation and that urea nitrogen may arise directly from aspartic acid

Ornithine is conjugated with benzoic acid in the fowl to form ornithuric acid (dibenzoyl ornithine) The reaction is analogous to the formation of hippuric acid from glycine and benzoic acid in other animals

10 Proline and Hydroxyproline

Experiments in which proline, labeled with deuterium and N^{15}, was fed to rats have conclusively demonstrated the conversion of this amino acid to glutamic acid, arginine, and ornithine [571] Previous *in vitro* studies had already indicated that the oxidation of L-proline led to the formation of α-ketoglutarate and glutamine [572, 573, 574] More recently, the complete oxidation of both L-proline and hydroxy L-proline by washed liver and kidney preparations fortified with adenosine triphosphate and magnesium ions was described [575] With a system deficient in cofactors, the formation of glutamic acid was demonstrated Neither pyrrolidone carboxylic acid nor α-amino-δ-hydroxyvaleric acid are believed to be intermediates in the conversion of proline to glutamic acid It has been suggested that the intermediate is glutamic semialdehyde, which could be oxidized to glutamic acid By analogy hydroxyproline might be expected to yield γ-hydroxyglutamic acid

The conversion of ornithine to proline and glutamic acid[576, 577] and of ornithine to arginine[578] has been demonstrated with deuterium-labeled ornithine in intact animals The interrelationship between proline, glutamic acid, and ornithine is therefore well established Further evidence of this relationship is derived from microbiological studies[579, 580, 581] and rat growth experiments [582] Hydroxyproline may be converted to proline, ornithine, or glutamate at a slow rate, or it is possible that hydroxyproline is metabolized by a different pathway There is evidence that hydroxyproline arises by oxidation of proline [571, 583] In *Escherichia coli*[584a] and *Neurospora crassa*[584b]

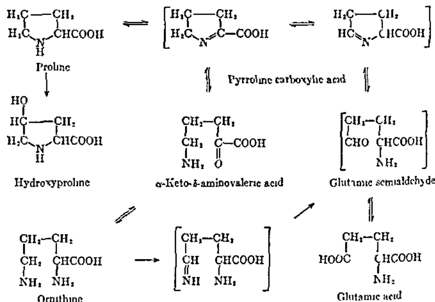
[571] M R Stetten and R Schoenheimer, *J Biol Chem* 153, 113 (1944)
[572] F Bernheim and M L C Bernheim, *J Biol Chem* 96, 325 (1932), 106, 79 (1934).
[573] H Weil-Malherbe, and H A Krebs, *J Biol Chem* 29, 2077 (1935)
[574] M Neber, *Z physiol Chem* 240, 70 (1936)
[575] J V Taggart and R B Krakaur, *J Biol Chem* 177, 641 (1949)
[576] M Roloff, S Ratner, and R Schoenheimer, *J Biol Chem* 136, 561 (1940)
[577] M R Stetten, *J Biol Chem* 189, 499 (1951)
[578] R F Clutton, R Schoenheimer, and D Rittenberg, *J Biol Chem* 132, 227 (1940)
[579] E L Tatum, *Federation Proc* 8, 511 (1949)
[580] R J Sirny, L T Cheng, and C A Elvehjem, *J Biol Chem* 190, 547 (1951)
[581] D W Hood and C M Lyman, *J Biol Chem* 185, 39 (1950)
[582] M Womack and W C Rose, *J Biol Chem* 171, 37 (1947)
[583] M R Stetten, *J Biol Chem* 181, 31 (1949)
[584a] H J Vogel and B D Davis, *J Am Chem Soc* 74, 109 (1952)
[584b] N. Good and H. K. Mitchell, *J Am Chem Soc* 74, 4952 (1952).

evidence supporting conversion of glutamic acid to proline via glutamic semialdehyde and Δ^1-pyrroline-5-carboxylic acid has been obtained

The action of D-amino acid oxidase on D-proline leads to α-keto-δ-amino-valeric acid which has been isolated as the 2,4-dinitrophenylhydrazone [584] The same product results from the oxidation of L-proline by L-oxidases (Section IV 3) and from the oxidation of D-ornithine by the D-oxidase [581]

Shemin and Rittenberg[585] found that ornithine isolated from rats fed N^{15}-labeled glycine contained equivalent concentrations of isotope in the α- and δ-amino groups This result may be explained by a shift of the α-amino group of ornithine to the δ-position by a mechanism not involving amino groups derived from other sources

A tentative metabolic scheme, describing the relationships between ornithine, glutamic acid, and proline, may be formulated:



11. Histidine

Although histidine is an essential dietary component for the rat,[586] the mouse,[587] the chick,[588, 589] and the dog,[590] it is not required for nitrogen

[584] H A Krebs, *Enzymologia* **7**, 53 (1939)
[585] D Shemin and D Rittenberg, *J Biol. Chem.* **158**, 71 (1945)
[586] W. C Rose and G J Cox, *J Biol Chem.* **61**, 747 (1924)
[587] J R Totter and C P Berg, *J Biol Chem* **127**, 375 (1939).
[588] H J Almquist and C R Grau, *J Nutrition* **28**, 325 (1944)
[589] H. J Almquist, *J Nutrition* **34**, 543 (1947)
[590] W C Rose and E. E Rice, *Science* **90**, 186 (1939).

equilibrium in normal, adult man,[591, 592] and it has been reported that it is probably not required by infants [212] These findings suggest that histidine may be synthesized by the tissues or the intestinal flora of man Studies by Broquist and Snell[593] on certain lactic acid bacteria suggest that the purine bases serve as precursors of histidine The possibility that histidine might be a precursor of the purine bases has not been supported by experimental evidence [594] Broquist and Snell[593] also found that imidazolepyruvic acid replaced histidine in supporting the growth of *L arabinosus* and *S faecalis* The presence of vitamin B_6 was required for the utilization of the keto acid, suggesting its conversion to histidine via transamination Recent studies on yeast by Levy and Coon[595] conclusively demonstrate that formate is a precursor of histidine These investigators found that formate furnished the 2-carbon of the imidazole ring, while glycine, labeled with C^{14} in the carboxyl group, did not contribute to radioactive histidine in growing yeast The purine bases do not contribute to histidine formation in this organism Histidinol may be a precursor of histidine in *E coli* [596]

The catabolism of histidine has received considerable attention The decomposition of histidine by liver was first observed by Edlbacher[597, 598] and by György and Röthler [599] The breakdown of histidine was considered to be catalyzed by a specific enzyme, histidase, which has been found in vertebrate liver and studied extensively in cat liver Histidase does not attack D-histidine, methylhistidine, imidazole, imidazolelactic acid, or urocanic acid [595, 600] The mechanism of histidase action remains to be completely elucidated The experimental findings indicate that the reaction is a hydrolytic one leading to splitting of the imidazole ring, an associated formation of 1 mole of ammonia, and loss of Van Slyke α-amino nitrogen. If the product of the reaction is treated with strong acid or alkali, 1 mole each of glutamate and formate, and an additional mole of ammonia appear. Edlbacher and Neber[601] and Walker and Schmidt[602] have formulated possible mechanisms of histidase action Edlbacher and Neber suggested for-

[591] W C Rose, W J Haines, D T Warner, and J E Johnson, *J Biol Chem* **188**, 49 (1951)

[592] A A Albanese, L E Holt, Jr , J E Frankston, and V Irby, *Bull Johns Hopkins Hosp* **74**, 251 (1944)

[593] H P Broquist and E E Snell, *J Biol Chem* **180**, 59 (1949)

[594] C Tesar and D Rittenberg, *J Biol Chem* **170**, 35 (1947)

[595] L Levy and M J Coon, *J Biol Chem* **192**, 807 (1951)

[596] H J Vogel, B D Davis, and E S Mingioli, *J Am Chem Soc* **73**, 1897 (1951)

[597] S Edlbacher, *Z physiol Chem* **157**, 106 (1926)

[598] S Edlbacher and J Kraus, *Z physiol Chem* **191**, 225 (1927), **195**, 267 (1931)

[599] P György and H Röthler, *Biochem Z* **173**, 334 (1926)

[600] F Leuthardt, in Sumner and Myrbäck The Enzymes, Academic Press, New York, 1951, Vol I, Part 2, p 1156

[601] S Edlbacher and M Neber, *Z physiol Chem* **225**, 261 (1934)

[602] A C Walker and C L A Schmidt, *Arch Biochem* **5**, 445 (1944)

ylglutamine ($COOHCHNH_2CH_2CH_2CONHCHO$) as the primary reaction product This was considered to undergo cyclic change involving the α-amino group Walker and Schmidt suggested that α-formamidinoglutamic acid ($COOHCH_2CH_2CHCOOH$) was the initial reaction product.

$$\begin{array}{c} COOHCH_2CH_2CHCOOH \\ | \\ NHCH{=}NH \end{array}$$

The conversion of histidine to urocanic acid by bacteria of the *typhosus coli* group was demonstrated by Raistrick.[603] Darby and Lewis[604] confirmed the formation of urocanic acid from histidine by *Bacillus paratyphosus* A, and also isolated urocanic acid from the urine of five of eight rabbits fed histidine The isolation of urocanic acid from the urine of animals had previously been reported,[605-611] although the presence of this compound was not a regular finding. It was suggested by some workers that urocanic acid might represent a relatively rare and unimportant side product of histidine metabolism [604, 612]

However, other investigators believe that urocanic acid represents a normal histidine metabolite Sera and Yada[613, 614] showed that histidine was converted to urocanic acid by liver preparations Similar results were obtained by Takeuchi.[615] It was found that liver preparations catalyzed the degradation of urocanic acid to optically inactive isoglutamine. Later, Sera and Aihara[616] isolated what they considered to be optically active formylisoglutamine (A) as a product of the enzymatic degradation of urocanic acid Oyamada[617] reported the isolation of optically inactive α-for-

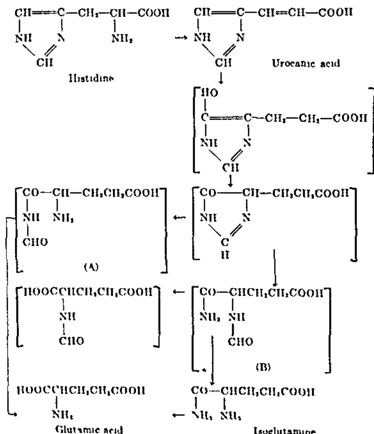
[illegible]

the Japanese workers, was demonstrated by Tabor and Hayaishi [618] These investigators found that cell-free extracts of *Pseudomonas fluorescens* catalyzed the quantitative conversion of L-histidine of L-glutamic acid, formic

603 H Raistrick, *Biochem J* **11**, 71 (1917)
604 W J Darby and H B Lewis, *J Biol Chem* **146**, 225 (1942).
605 M Jaffe, *Ber* **7**, 1669 (1874), **8**, 811 (1875)
606 A Hunter, *J Biol Chem* **11**, 537 (1912)
607 R L Swain, *Am J Physiol* **13**, 30 (1905)
608 A Hunter and M H Givens, *J Biol Chem* **8**, 449 (1910)
609 K Kotake and M Konishi, *Z physiol Chem* **122**, 230 (1922)
610 M Konishi, *Z physiol Chem* **122**, 237 (1922)
611 [illegible]
612 [illegible]
613 [illegible] cited in ref 614.
614 [illegible]
615 [illegible]
616 [illegible]
617 [illegible]
618 [illegible]

acid, and 2 moles of ammonia Subsequently, Tabor *et al.*[419] obtained unequivocal evidence for this conversion using labeled histidine. These studies indicate that the γ-nitrogen atom of the imidazole ring is the precursor of the α-amino group of glutamic acid and that the α-amino group of histidine is liberated as ammonia during the "histidase" reaction Using histidine labeled with C^{14} in the 2 position of the ring, it was found that this carbon atom is the precursor of the formate Furthermore, the conversion of C^{14}-labeled histidine to radioactive urocanic acid was demonstrated

Although the mechanism of the histidine → glutamic acid conversion requires further clarification, the following scheme may be tentatively considered



[419] H Tabor, A Mehler, O Hayaishi, and J White, *J. Biol Chem* **196**, 121 (1952)

In this scheme, the possibility of the formation of α-N-formylglutamic acid is considered, since it is possible that the α-amide may be hydrolyzed prior to the splitting of formate. It should also be noted that unequivocal proof of structure of the formylisoglutamine compound is as yet lacking.

The degradation of isotopic histidine in higher animals has also been studied No *evidence of a selective conversion of imidazole nitrogen to* glutamic acid in rats was observed by Tesar and Rittenberg,[594] although these studies did not exclude a histidine → glutamate conversion. Mehler and Tabor,[619a] extending the studies of Tabor *et al.*[619] to mammalian tissues, have concluded that the urocanic acid pathway is the major or exclusive route of histidine degradation in guinea pig liver. Similar conclusions have *been independently reached by Abrams and Borsook*[619b] *The enzyme re*sponsible for deamination of histidine to urocanic acid (histidine α-deaminase) has been investigated in cat liver.[619c] Carbon 2 of the imidazole ring is also the precursor of formate in animal tissues.[619d, 619e, 619f]

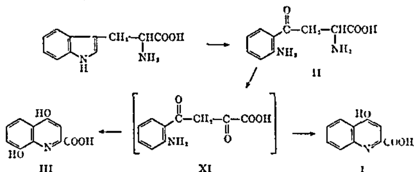
12 Tryptophan

Relatively *early studies* revealed that kynurenic acid (I) was excreted in the urine of animals after the feeding of tryptophan.[620-622] The structure of this compound was established in 1914 by Homer.[623] It was also found in bile,[624, 625] and its formation was observed in liver perfusion experiments with tryptophan and indolepyruvic acid [626] In 1925, Matsuoka and Yoshimatsu[78] discovered kynurenine (II) in the urine of rabbits fed large amounts of tryptophan The determination of structure and the synthesis of L-kynurenine was accomplished by Butenandt *et al*[79] In 1937, Masajo[627, 628] isolated xanthurenic acid (III) from the urine of rats fed fibrin. Subsequently, it was isolated from the urine of vitamin B_6-deficient rats and from the urine of other animals

The conversion of kynurenine to xanthurenic acid in the rat has been demonstrated It has been concluded that both xanthurenic and kynurenic

619a A Mehler and H Tabor, *Federation Proc.* **11**, 374 (1952)
619b A Abrams and H Borsook, *J Biol Chem* **198**, 205 (1952)
619c D A Hall, *Biochem J* **51**, 499 (1952)
619d J C Reid and M O Landefeld, *Arch. Biochem Biophys.* **34**, 219 (1951)
619e M Toporek, L L Miller, and W F Bale, *J Biol Chem* **198**, 839 (1952)
619f D B Sprinson and D Rittenberg, *J Biol Chem* **198**, 655 (1952)
620 J Liebig, *Ann* **86**, 125 (1853)
621 [illegible]
622 [illegible] 1933)
623 [illegible]
624 [illegible] *hem* **123**, 151 (1938)
625 [illegible] 1 (1931)
626 [illegible] . 175 (1922)
627 [illegible]
628 [illegible]

acids are derived from kynurenine, but it would appear that neither of these acids may lie on the major pathway of tryptophan metabolism:



A relationship between nicotinic acid and tryptophan was demonstrated in 1945 by Krehl *et al*,[629] who found that rats fed rations deficient in nicotinic acid would grow if given tryptophan The existence of this relationship has been amply confirmed (see, for example, refs 630 and 631) It is interesting to note that Goldberger had reported in 1922 that the administration of tryptophan to pellagrins had some beneficial effect on this disease [632] Other studies[633] indicated that administration of tryptophan to rats resulted in increased urinary excretion of N^1-methylnicotinamide (XII) The conversion of tryptophan to nicotinic acid has been shown in studies on a number of species (cf ref 628).

A large body of evidence has accumulated on the metabolic steps involved in the conversion of tryptophan to nicotinic acid Studies with *Neurospora*—[634–643] indicated that L-kynurenine was an intermediate in this con-

[629] W A Krehl, L J Teply, P S Sarma, and C A Elvehjem, *Science* **101**, 489 (1945), *J Nutrition* **31**, 85 (1946)
[630] H Spector, *J Biol Chem* **173**, 659 (1948)
[631] H P Sarett, *J Biol Chem* **182**, 659, 671, 679, 691 (1950)
[632] J Goldberger and W F Tanner, *Pub Health Reports* **37**, 462 (1922)
[633] F Rosen, J W Huff, and W A Perlzweig, *J Biol Chem* **163**, 343 (1946), **167**, 511 (1947)
[634] G W Beadle, H K Mitchell, and J F Nye, *Proc Natl Acad Sci U S* **33**, 155 (1947)
[635] H K Mitchell and J F Nye, *Proc Natl Acad Sci U S* **34**, 1 (1948)
[636] F A Haskins and H K Mitchell, *Proc Natl Acad Sci U S* **35**, 500 (1949)
[637] D Bonner and C Yanofsky, *Proc Natl Acad Sci U S* **35**, 576 (1949)
[638] D Bonner and G W Beadle, *Arch Biochem* **11**, 319 (1946)
[639] C Yanofsky, E Wasserman, and D Bonner, *Science* **111**, 61 (1950)
[640] J F Nye and H K Mitchell, *J Am Chem Soc* **70**, 1847 (1948).
[641] J F Nye H K Mitchell, E Leifer, and W H Langham, *J. Biol. Chem* **179**, 783 (1949)
[642] D Bonner *Proc Natl Acad Sci U S* **34**, 5 (1948)
[643] D M Bonner and E Wasserman, *J Biol Chem* **185**, 69 (1950).

version These investigations suggested that kynurenine (II) was oxidized to 3-hydroxykynurenine (IV), which in turn was converted to 3-hydroxyanthranilic acid (V). Kynurenine may also lead to anthranilic acid (VI), which is converted to indole (VII) and thence, by synthesis involving serine, to tryptophan 3-Hydroxyanthranilic acid is converted to nicotinic acid (VIII), and there is evidence that quinolinic acid (IX) is an intermediate in this reaction Nutritional and isotopic studies in animals suggest the same general pathway. 3-Hydroxyanthranilic acid is a precursor of nicotinic acid in the rat.[644] Studies by Heidelberger *et al.*[645, 646] demonstrated the conversion of tryptophan to nicotinic acid in the rat, and that carbon atom 3 of the indole ring of tryptophan becomes the carboxyl carbon atom of nicotinic acid These investigators also showed that tryptophan was converted into kynurenine in rabbits, and kynurenic acid in dogs The β-carbon atom of tryptophan becomes the β-carbon atom of kynurenine and the 3-carbon atom of kynurenic acid The tryptophan side chain does not appear in the nicotinic acid molecule.[645, 647] Evidence from *Neurospora* studies *in vivo* animal experiments and *in vitro* work implicates quinolinic acid as an intermediate between 3-hydroxyanthranilic acid and nicotinic acid.

Schayer,[648] using tryptophan labeled with N^{15} in the ring, observed the conversion of tryptophan to kynurenine, kynurenic acid, and xanthurenic acid in rats and rabbits This worker found no evidence for the conversion of the tryptophan pyrrole ring to hemin or of N^{15}-labeled indole to tryptophan in the rat The evidence also indicated a conversion of D-tryptophan to its enantiomorph, and that the ring nitrogen is utilized for the synthesis of other amino acids.

The conversion of monomethyl-L-tryptophan to nicotinic acid and N^1-methylnicotinamide by the rat was recently shown by Sung and Tung[649]

The mechanism of the tryptophan → kynurenine conversion was studied with a soluble liver enzyme system by Mehler and Knox.[650, 651] It was shown that tryptophan was converted by means of a coupled system consisting of a peroxidase and an oxidase, to formylkynurenine (X), which was hydrolyzed to formate and kynurenine by the enzyme formylase Formyl-

[644] H K Mitchell, J F Nye, and R D Owen, *J Biol Chem* **175**, 433 (1948)

[645] C Heidelberger, M E Gullberg, A F Morgan, and S Lepkovsky, *J. Biol Chem.* **179**, 143 (1949)

[646] C Heidelberger, E P Abraham, and S Lepkovsky, *J. Biol Chem* **179**, 151 (1949)

[647] J M Hundley and H W Bond, *Arch Biochem* **21**, 313 (1949)

[648] R W Schayer, *J Biol Chem* **187**, 777 (1950)

[649] S-C Sung and T-C Tung, *J Biol Chem* **186**, 637 (1951)

[650] W E Knox and A. H. Mehler, *J Biol Chem* **187**, 419 (1950)

[651] A. H Mehler and W. E Knox, *J Biol Chem* **187**, 431 (1950)

kynurenine is probably an intermediate in tryptophan oxidation in certain bacteria.[651a]

Early work had suggested a role of vitamin B_6 in tryptophan metabolism. Later it was found that vitamin B_6 deficiency was associated with an impaired conversion of tryptophan and kynurenine to nicotinic acid.[652, 653] It is now believed that vitamin B_6 in the form of pyridoxal phosphate is involved in the conversion of kynurenine to anthranilic acid and alanine by the enzyme kynureninase.[654-657] The mechanism of this reaction[656] may be explained in terms of an initial transamination between pyridoxal phosphate and kynurenine to yield *o*-aminobenzoylpyruvate (XI). By analogy with the hydrolysis of benzoylpyruvate,[407] *o*-aminobenzoylpyruvate might be expected to yield anthranilic acid pyruvate (Section IV 7). The latter could form alanine by transamination. The formation of some kynurenic acid as a side product of this reaction is compatible with the intermediate participation of *o*-aminobenzoylpyruvate, since the latter compound would be expected to yield kynurenic acid by spontaneous cyclization. A similar mechanism may exist for the conversion of 3-hydroxykynurenine to 3-hydroxyanthranilic acid.

Sanadi and Greenberg[658] have suggested that *o*-amino-*m*-hydroxybenzoylacetic acid may be formed by decarboxylation of *o*-amino-*m*-hydroxybenzoylpyruvate. The β-keto acid might decompose to hydroxyanthranilic acid and an acetyl derivative.

Possible metabolic pathways of tryptophan are given in the scheme on the following page.

The anaerobic degradation of tryptophan to indole in bacteria was first reported by Hopkins and Cole.[659] In the presence of oxygen, indoleacetic acid was also formed. Subsequent studies showed that tryptophan was broken down to indole by a specific enzyme, tryptophanase.[660, 661] Early work and several suggested mechanisms of the reaction have been discussed by Happold.[661] Recently, Wood, Gunsalus, and Umbreit[662] prepared the enzyme from *E. coli* and demonstrated that pyridoxal phosphate was re-

[651a] R. Y. Stanier, D. Hayaishi, and M. Tsuchida, *J. Bact.* 62, 355 (1951).
[652] R. S. Schweigert and P. B. Pearson, *J. Biol. Chem.* 168, 555 (1947).
[653] Y. Kotake and Nakayama, *Z. physiol. Chem.* 270, 76 (1941).
[654] A. E. Braunstein, E. V. Goryachenkova, and T. S. Paskhina, *Biokhimiya* 14, 163 (1949).
[655] O. Wiss, *Helv. Chim. Acta* 32, 1694 (1949).
[656] C. E. Dalgliesh, W. E. Knox, and A. Neuberger, *Nature* 168, 20 (1951).
[657] O. Hayaishi and R. Y. Stanier, *J. Biol. Chem.* 195, 735 (1952).
[658] D. R. Sanadi and D. M. Greenberg, *Arch. Biochem.* 25, 323 (1950).
[659] F. G. Hopkins and S. W. Cole, *J. Physiol.* 29, 451 (1903).
[660] F. C. Happold and L. Hoyle, *Biochem. J.* 29, 1918 (1935).
[661] F. C. Happold, *Advances in Enzymol.* 10, 51 (1950).
[662] W. A. Wood, I. C. Gunsalus, and W. W. Umbreit, *J. Biol. Chem.* 170, 313 (1947).

quired for its activity The reaction catalyzed by the purified enzyme yielded equimolar amounts of indole, ammonia, and pyruvate from tryptophan The system did not absorb oxygen, nor deaminate alanine or serine Certain of these findings have been confirmed.[663] Dawes *et al* [664] presented evidence suggesting that alanine was a product However, amination of pyruvate by other systems was not excluded. The exact reaction mechanism has not yet been discovered. Recently, it was reported that indole and pyruvate catalyzed the tryptophanase reaction, and it was suggested that the reaction was autocatalytic in nature [665]

[663] E. A. Dawes and F. C Happold, *Biochem J* **44**, 349 (1949)
[664] E. A Dawes, J. Dawson, and F. C. Happold, *Biochem. J.* **41**, 426 (1947)
[665] E. Beerstecher, Jr. and E J. Edmonds, *J Biol. Chem* **192**, 497 (1951).

The synthesis of tryptophan in *Neurospora* involves a reaction utilizing indole and serine[666, 667, 668] Pyridoxal phosphate has been shown to be necessary for the synthesis of tryptophan in *Neurospora*[669] and in *L arabinosus*

Recently Williams and Elvehjem[670] observed that tryptophan deficiency in the rat resulted in decreased activity of several liver enzymes Continuation of these researches[671, 672] has led to the finding that dietary tryptophan was necessary for the synthesis of liver pyridine nucleotides.

13 Phenylalanine and Tyrosine

Knowledge of the metabolic pathways of tyrosine and phenylalanine has been advanced by the study of certain "inborn errors of metabolism" in man Alcaptonuria, a disorder in which homogentisic acid[673, 674] is excreted in the urine, has been known for at least several centuries, although it was first carefully described about 90 years ago[675, 676] The condition is inherited and is transmitted as a recessive Mendelian character Individuals afflicted with this disorder are usually normal in other respects The disease is frequently noted early in life as evidenced by the darkening of urine or staining of diapers remaining in contact with the air Homogentisic acid occurs in other body fluids,[677] and later in life alcaptonuric individuals may suffer a darkening of tendons and cartilage (ochronosis)[676] When phenylalanine or tyrosine is administered to alcaptonurics, extra homogentisic acid is excreted in the urine However, the administration of these amino acids, or of homogentisic acid to normal individuals, results in utilization of these compounds, and homogentisic acid excretion is not observed Normal animals oxidize homogentisic acid (as well as phenylalanine and tyrosine) to products including acetoacetate The formation of acetoacetate from homogentisic acid was observed in liver perfusion experiments by Embden *et al*[678] The administration of very large amounts of phenylalanine and

666 E. L Tatum, D Bonner, and G W Beadle, *Arch Biochem* 3, 477 (1944)
667 E. L Tatum and D Bonner, *J Biol Chem* 151, 349 (1943)
668 E. L Tatum and D Bonner, *Proc Nat Acad Sci U S* 30, 30 (1944)
669 W W Umbreit, W A Wood, and I C Gunsalus, *J Biol Chem* 165, 731 (1946)
670 J N Williams, Jr and C A Elvehjem, *J Biol Chem* 183, 539 (1950)
671 J N Williams, Jr, P Feigelson, and C A Elvehjem, *J Biol Chem* 187, 597 (1950)
672 J N Williams, Jr, P Feigelson, S S Shahinian, and C A Elvehjem, *J Biol Chem* 189, 659 (1951)
672a M. Duncan and H P Sarett, *J Biol Chem* 193, 317 (1951).
673 J Marshall, *Am J Pharm* 59, 131 (1887)
674 M Wolkow and E Baumann, *Z physiol Chem* 15, 228 (1891)
675 C Boedeker, *Z f rat Med* 7, 130 (1859), *Ann* 117, 82 (1861).
676 A E Garrod, Inborn Errors of Metabolism, Oxford Medical Publications, London, 1923
677 F Lanyar and H Lieb, *Z physiol Chem.* 203, 135 (1931)
678 G Embden, H Salomon, and F Schmidt, *Biochem Z* 65, 301 (1913)

tyrosine to experimental animals has also been found to lead to homogentisic acid excretion.[679-684] The accumulated evidence is compatible with the concept that homogentisic acid is a normal metabolite in phenylalanine and tyrosine metabolism. *Its appearance in the urine may be attributed to* a defect in the mechanism responsible for its oxidation.

Phenylalanine may be converted to phenylpyruvic acid, phenyllactic acid, or by decarboxylation of the keto acid to phenylacetic acid. Another pathway, to be discussed later, may lead to adrenaline. The primary catabolic route of phenylalanine appears to involve oxidation to tyrosine. Several lines of evidence are available. It was observed by Embden and Baldes[685] that tyrosine was formed from phenylalanine in liver perfusion experiments. Administration of phenylalanine to a person with tyrosinosis resulted in the urinary excretion of tyrosine.[686] Phenylalanine promotes the growth of rats fed tyrosine-deficient rations (Section III.1). In ascorbic acid-deficient premature infants administration of phenylalanine leads to tyrosine excretion.[687, 688] Unequivocal evidence for the conversion of phenylalanine to tyrosine in rats was obtained in studies with ring-labeled (deuterium) phenylalanine.[689] Recently, the enzymatic oxidation of phenylalanine to tyrosine by an isolated liver enzyme system has been reported.[690]

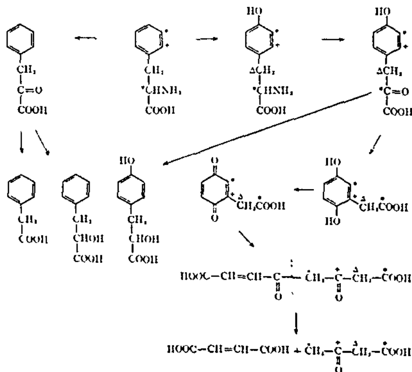
The conversion of phenylalanine to acetoacetate has been examined with the aid of carbon isotopes by Schepartz and Gurin.[691] It was shown that (*a*) the α-carbon atom of phenylalanine was the precursor of the carboxyl carbon atom of acetoacetate, (*b*) the carbonyl carbon atom of acetoacetate was derived from carbon atom 2 of the ring, and (*c*) the carbon atom 1 or 3 of the ring is the precursor of the terminal carbon atom of acetoacetate. It has also been found that the β-carbon atom of tyrosine becomes the α-carbon atom of acetoacetate.[692, 693, 694] These findings may be explained in terms of a shift of the side chain during oxidation. Other studies[695]

showed that the catabolism of tyrosine and phenylalanine led to the formation of two 4-carbon units, one a ketone body, and the other, malic acid (or its precursor)

It was shown in independent studies[696, 697, 698] that transamination of tyrosine with α-ketoglutarate to yield *p*-hydroxyphenylpyruvate is the initial step in tyrosine oxidation The oxidation of *p*-hydroxyphenylpyruvate to homogentisate may involve 2,5-dihydroxyphenylpyruvate as an intermediate [696] This keto acid could be decarboxylated to yield homogentisate However, decarboxylation may precede or occur simultaneously with shift of the side chain The mechanism of this interesting reaction requires investigation

Ravdin and Crandall[410] have shown that homogentisic acid is oxidized by liver preparations to 4-fumarylacetoacetic acid A straight-chain 8-carbon dicarboxylic acid intermediate had been suggested by Lerner [675] Fumarylacetoacetate was hydrolyzed to fumarate and acetoacetate,[410] possibly by the same system which was previously reported to hydrolyze 2,4- and 3,5-diketo acids (Section IV 7)

A postulated pathway of the conversion of phenylalanine to acetoacetate is as follows



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679 [illegible] (1903).
680 [illegible]
681 [illegible] 938).
682 [illegible]
683 [illegible]
684 [illegible] 0, 153 (1941)
685 G. Embden and K. Baldes, *Biochem. Z.* 55, 301 (1913)
686 G. Medes, *Biochem. J.* 26, 917 (1932)
[illegible] 1941)

3,4-Dihydroxyphenylalanine is oxidized by an incompletely understood mechanism to melanin by tyrosinase and dopa oxidase.[704, 705] The separate identity of these enzymes in mammalian tissues has been questioned[706]

14 Methionine, Cysteine, and Cystine

The conversion of both isomers of methionine to α-keto-γ-methiolbutyric acid by the respective amino acid oxidases has been demonstrated by isolation of the keto acid hydrazone[707, 708] The further pathway of degradation of this keto acid, which may also arise from L-methionine as a product of transamination, has not yet been discovered It may, however, be reaminated Thus, the ability of D-methionine to support growth may be ascribed to oxidation to α-keto-γ-methiolbutyrate and subsequent transamination to L-methionine. The possibility that α-keto-γ-methiolbutyric acid may take part in transmethylation reactions should be investigated

An important metabolic pathway of L-methionine is demethylation to homocysteine With protein diets deficient in methionine, D- or L-homocystine supports the growth of rats[709] It is probable that homocysteine and homocystine are interconvertible in the body, as in the case of cysteine and cystine[710] With certain methionine deficient diets, homocystine does not promote growth,[711, 712] unless methyl groups are supplied, as for instance, by the addition of choline to the diet[713] The demethylation of methionine to homocysteine results in the liberation of a "labile methyl group" capable of methylating other compounds, e g , guanidoacetic acid may accept a methyl group to form creatine[714] Another example of "transmethylation" is the methylation of homocysteine to methionine by choline That the methyl group is transferred intact was unequivocally demonstrated by du Vigneaud and his collaborators[715] It was shown that there was no loss of deuterium from a carbon-labeled methyl group during the transfer. It was also found

[704] H S Raper, *Biochem J* **21**, 89 (1927), *J Chem Soc* **1938**, 125
[705] H S Mason, *J Biol Chem* **172**, 83 (1948), **181**, 803 (1949)
[706] A B Lerner, T B Fitzpatrick, E Calkins, and W H Summerson, *J Biol Chem* **178**, 185 (1949)
[707] D E Green, D H Moore, V Nocito, and S Ratner, *J Biol Chem* **156**, 383 (1946)
[708] H Waelsch and E Borek, *J Am Chem Soc* **61**, 2252 (1939)
[709] H M Dyer and V du Vigneaud, *J Biol Chem* **109**, 477 (1935)
[710] P Desnuelle and C Fromageot, *Compt rend* **216**, 350 (1943)
[711] V du Vigneaud, H M Dyer, and M Kies, *J Biol Chem* **130**, 325 (1939).
[712] W C Rose and E E Rice, *J Biol Chem.* **130**, 305 (1939)
[713] V du Vigneaud, J P Chandler, A W Moyer, and D M Keppel, *J. Biol Chem* **131**, 57 (1939)
[714] V du Vigneaud, J P Chandler, M Cohn, and G B Brown, *J Biol Chem* **134**, 787 (1940).
[715] E B Keller, J R Rachele and V du Vigneaud, *J Biol Chem.* **177**, 233 (1949)

Ascorbic acid-deficient animals fed large amounts of phenylalanine and tyrosine excrete *p*-hydroxyphenylpyruvic acid in the urine. The excretion of this keto acid is reduced by administration of ascorbic acid.[679-684] *Premature infants deficient in ascorbic acid and full-term infants fed a high* protein ration were found to excrete *p*-hydroxyphenylpyruvic acid and *p*-hydroxyphenyllactic acid. This phenomenon, also, was reversed by ascorbic acid [687, 688] A number of investigators found that ascorbic acid was involved in the *in vitro* oxidation of tyrosine (see, for example, ref. 699) Recently, Knox and LeMay-Knox[696] reported that catalytic amounts of this vitamin were required for the oxidation of *p*-hydroxyphenylpyruvate by a soluble liver enzyme system. An observation of some interest is that of Woodruff *et al.*,[700] who were able to prevent hydroxyphenyluria (but not scurvy) in guinea pigs fed an ascorbic acid deficient diet, by administration of pteroylglutamic acid.[700]

In tyrosinosis, administration of tyrosine resulted in excretion of *p*-hy-
4-dihy-
iciency
a de-
lic dis-
order, phenylpyruvic oligophrenia, is characterized by excretion of phenylpyruvic acid and mental deficiency. Administered phenylalanine is partly excreted as the keto analogue Although the exact mechanism of the "error" is not known, it probably represents a deficiency in oxidation of phenylalanine to tyrosine [700a]

Another oxidative pathway of phenylalanine and tyrosine may lead to the formation of adrenaline Gurin and Delluva[701] have demonstrated that phenylalanine is a precursor of adrenaline in the rat, and that oxidation of the ring occurs with the original side-chain intact The conversion may involve decarboxylation to 3,4-dihydroxyphenylethylamine (Section IV 4)

other aromatic amino acids [702, 703]

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696 M LeMay-Knox and W E Knox, *Biochem J* 49, 686 (1951)
697 *Chem* 190, 245 (1951)
698
699 4, 645 (1951)
700 C W Woodruff, M E ... and W J Darby, *J Biol Chem* 178, 861 (1949)
700a G A Jervis, *J Biol Chem* 169, 651 (1947)
701 S Gurin and A M Delluva, *J Biol Chem* 170, 545 (1947)
702 B D Davis, *Experimentia* 6, 41 (1950)
703 B D Davis, *J Biol Chem.* 191, 315 (1951)

3,4-Dihydroxyphenylalanine is oxidized by an incompletely understood mechanism to melanin by tyrosinase and dopa oxidase.[704, 705] The separate identity of these enzymes in mammalian tissues has been questioned [706]

14. Methionine, Cysteine, and Cystine

The conversion of both isomers of methionine to α-keto-γ-methiolbutyric acid by the respective amino acid oxidases has been demonstrated by isolation of the keto acid hydrazone [707, 708] The further pathway of degradation of this keto acid, which may also arise from L-methionine as a product of transamination, has not yet been discovered It may, however, be reaminated Thus, the ability of D-methionine to support growth may be ascribed to oxidation to α-keto-γ-methiolbutyrate and subsequent transamination to L-methionine The possibility that α-keto-γ-methiolbutyric acid may take part in transmethylation reactions should be investigated

An important metabolic pathway of L-methionine is demethylation to homocysteine. With protein diets deficient in methionine, D- or L-homocystine supports the growth of rats [709] It is probable that homocysteine and homocystine are interconvertible in the body, as in the case of cysteine and cystine [710] With certain methionine deficient diets, homocystine does not promote growth,[711, 712] unless methyl groups are supplied, as for instance, by the addition of choline to the diet [713] The demethylation of methionine to homocysteine results in the liberation of a "labile methyl group" capable of methylating other compounds, e g , guanidoacetic acid may accept a methyl group to form creatine [714] Another example of "transmethylation" is the methylation of homocysteine to methionine by choline That the methyl group is transferred intact was unequivocally demonstrated by du Vigneaud and his collaborators [715] It was shown that there was no loss of deuterium from a carbon-labeled methyl group during the transfer It was also found

[704] H S Raper, *Biochem J* 21, 89 (1927), *J Chem Soc* 1938, 125
[705] H S Mason, *J Biol Chem* 172, 83 (1948), 181, 803 (1949)
[706] A B Lerner, T B Fitzpatrick, E Calkins, and W H Summerson, *J Biol Chem* 178, 185 (1949)
[707] D E Green, D H Moore, V Nocito, and S Ratner, *J Biol Chem* 156, 383 (1946)
[708] H Waelsch and E Borek, *J Am Chem Soc* 61, 2252 (1939)
[709] H M Dyer and V du Vigneaud, *J Biol Chem*. 109, 477 (1935).
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[713] V du Vigneaud, J P Chandler, A W. Moyer, and D M. Keppel, *J. Biol. Chem*. 131, 57 (1939)
[714] V du Vigneaud, J P Chandler, M Cohn, and G B Brown, *J. Biol. Chem* 134, 787 (1940).
[715] E B Keller, J R Rachele, and V du Vigneaud, *J. Biol Chem* 177, 233 (1949)

that the methyl group of methionine was ultimately oxidized to carbon dioxide.[716] It is probable that formate is an intermediate in this oxidation.

In the original report of du Vigneaud *et al.*[713] describing studies in which rats were given a methionine-deficient ration containing homocystine, the authors noted that occasional animals showed some growth, even when choline was not added to the diet. Similar findings were obtained later by others,[717, 718] and it was found that growth in the absence of choline was abolished when sulfasuxidine was included in the diet.[719] Subsequent studies showed that labile methyl groups were synthesized either in the body tissues or by the intestinal flora.[720] Conclusive evidence that the synthesis of methyl groups occurred in the tissues was obtained with germ-free rats.[721]

A relationship between vitamin B_{12}, folic acid, and methionine became apparent as a result of several approaches.[722-728] It was observed that methionine and vitamin B_{12} were interchangeable under certain conditions for the growth of *E. coli*.[724] Vitamin B_{12} and folic acid exert a sparing action on choline in protecting rats and chicks against renal damage and perosis, respectively.[725] Stekol and Weiss[726] observed growth of young rats on "labile methyl"-free diets containing homocystine (or homocysteine) and vitamin B_{12}. Bennett[727] found that rats grew on a "labile methyl"-free diet containing folic acid and vitamin B_{12}. Jukes *et al.*[728] found that vitamin B_{12}-deficient chicks on a methionine-free diet could not utilize homocystine (or homocysteine) and betaine for growth unless the diet was supplemented with vitamin B_{12}. Recently it was demonstrated that labile methyl groups can be synthesized *in vivo* and *in vitro* by reduction of formate.[729-731] Livers from folic acid-deficient chicks exhibit a reduced ability to oxidize choline and to

716 C. G. Mackenzie, J. P. Chandler, E. B. Keller, J. R. Rachele, N. Cross, D. B. Melville, and V. du Vigneaud, *J. Biol. Chem.* **169**, 757 (1947), **180**, 99 (1949).

717 G. Toennies, M. A. Bennett, and G. Medes, *Growth* **7**, 251 (1943).

718 M. A. Bennett, G. Medes, and G. Toennies, *Growth* **8**, 59 (1944).

719 M. A. Bennett, *Federation Proc.* **4**, 83 (1945).

720 V. du Vigneaud, S. Simmonds, J. P. Chandler, and M. Cohn, *J. Biol. Chem.* **159**, 755 (1945).

721 V. du Vigneaud, C. Ressler, and J. R. Rachele, *Science* **112**, 267 (1950).

722 H. R. Bird, M. Rubin, and A. C. Groschke, *J. Nutrition* **33**, 319 (1947).

723 M. B. Gillis and L. C. Norris, *J. Biol. Chem.* **179**, 487 (1949).

724 W. Shive, cited in ref. 721.

725 A. E. Schaefer, W. D. Salmon, D. R. Strength, and D. H. Copeland, *J. Nutrition* **40**, 95 (1950).

726 J. A. Stekol and K. Weiss, *J. Biol. Chem.* **186**, 343 (1950).

727 M. A. Bennett, *J. Biol. Chem.* **187**, 751 (1950).

728 T. H. Jukes, E. L. R. Stokstad, and H. P. Broquist, *Arch. Biochem.* **25**, 453 (1950).

729 W. Sakami, *J. Biol. Chem.* **187**, 369 (1950).

730 W. Sakami and A. D. Welch, *J. Biol. Chem.* **187**, 379 (1950).

731 P. Berg, *J. Biol. Chem.* **190**, 31 (1951).

form methionine from homocysteine.[732] Studies by Mackenzie[733] have shown that formaldehyde as well as formate is formed in the oxidation of methyl groups in rats The 1-carbon fragment involved in methyl group synthesis may be formate, formaldehyde, or a derivative of one of these. It may be concluded that labile methyl groups, whether synthesized in the body or of dietary origin, are transferred intact in experimental animals, and in man [734] It has recently been reported that subcutaneously administered deuterium labeled C^{14} formate in the rat entered the methyl group of choline with no detectable loss of its hydrogen.[734a] The exact mechanism of the synthesis of methyl groups and the role of vitamin B_{12} and folic acid in this process are as yet not known

It is of interest that the concept of methylation was stated as early as 1894 by Hofmeister[735] and that this author suggested the possibility of transmethylation. A number of transmethylation reactions have been demonstrated *in vitro* Borsook and Dubnoff observed formation of methionine from homocysteine and choline or betaine in liver preparations [736] There is evidence that choline is converted to betaine prior to transmethylation [737] Dimethylthetin and dimethyl-β-propiothetin also serve as methyl donors for homocysteine [738] Transmethylation of nicotinamide[739], [740] guanidoacetic acid,[741, 742] glycine,[743] and ethanolamine[744] by methionine, and of guanidoacetic acid[745] and noradrenaline[746] by choline have been observed Keller *et al* [747] have shown the conversion of methionine methyl to adrenaline methyl *in vivo* Borsook and Dubnoff noted two categories of methyl transfer reactions One type requires oxygen, e g , methylation of guanidoacetic acid and nicotinamide by methionine In the second category is

[732] J S Dinning, C K Keith, and P L Day, *J Biol Chem* **189**, 515 (1950)
[733] C G Mackenzie, *J Biol Chem* **186**, 351 (1950)
[734] S Simmonds and V du Vigneaud, *J Biol Chem* **146**, 685 (1942)
[734a] C Ressler, J R Rachele, and V du Vigneaud, *J Biol. Chem* **197**, 1 (1952)
[735] H Hofmeister, *Arch exptl Path Pharmakol* **33**, 198 (1894)
[736] H Borsook and J W Dubnoff, *J Biol Chem* **160**, 635 (1945) **169**, 247 (1947)
[737] J A Muntz, *J Biol Chem* **182**, 489 (1950)
[738] Dubnoff J W , and Borsook, H *J Biol Chem* **176**, 789 (1948)
[739] W A Perlzweig, M L C Bernheim, and F Bernheim, *J Biol Chem* **150**, 401 (1943)
[740] P Ellinger, *Biochem J* **42**, 175 (1948)
[741] H Borsook and J W Dubnoff, *J Biol Chem* **132**, 559 (1940), **134**, 635 (1940)
[742] T L Sourkes, *Arch Biochem* **21**, 265 (1949)
[743] H K Barrenscheen, and T von Valyi-Nagy, *Z physiol Chem*, **277**, 97 (1942)
[744] G Steenholt, *Acta Physiol Scand* **11**, 294 (1946), **14**, 340 (1947)
[745] H K Barrenscheen and J Pany, *Z physiol. Chem* **233**, 78 (1948)
[746] E Bülbring, cited in Sourkes, T L *in* Sumner and Myrbäck, The Enzymes, Academic Press, 1950, Vol I, Part 2, p 1068
[747] E B Keller R A Boissonnas, and V du Vigneaud, *J Biol. Chem*, **183**, 627 (1950)

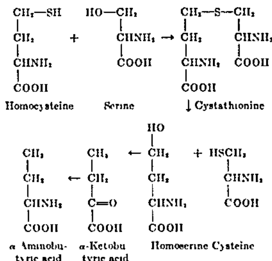
included the methylation of homocysteine by betaine or choline Reactions of the second type proceed without oxygen and persist after the cellular structure of the tissue is destroyed. High-energy phosphate is required for the methylation of guanidoacetic acid and noradrenalin. Recent evidence suggests that adenosine triphosphate is required to activate methionine prior to transmethylation[748] Transmethylation is clearly a significant biological phenomenon, and the mechanisms involved deserve further study.

The metabolism of homocysteine may involve oxidation to homocystine, oxidation to homocysteic acid,[749] desulfuration to hydrogen sulfide, or transsulfuration leading to cysteine Homocystine may be oxidized to sulfate by a mechanism as yet obscure Evidence for the occurrence of deamination to the corresponding α-keto acid is as yet lacking[750] The desulfuration *of homocysteine by the enzyme homocysteine desulfurase was first* noted by Fromageot and Desnuelle.[751] The enzyme is present in the liver, kidney, and pancreas of higher animals It has also been found in *P. morganii* where it leads to the formation of hydrogen sulfide, ammonia, and α-ketobutyrate[752] The inactivated *P. morganii* preparation was restored to activity by pyridoxal phosphate.

Homocysteine may also transsulfurate with serine to yield cysteine. The conversion of methionine sulfur to cystine sulfur was shown by Tarver and Schmidt[753] and later by du Vigneaud *et al.*[754] Brand *et al.*[755] noted that homocysteine sulfur administered to a cystinuric patient led to extra urinary cystine. Brand *et al.*[756] suggested a transfer of sulfur from homocysteine to cysteine via the intermediate, S-(β-amino-β-carboxyethyl)-homocysteine (later designated cystathionine) The studies of du Vigneaud and associates[757-762] have provided the evidence for this mechanism and further

748 G. L. Cantoni, *J Biol. Chem.* **189**, 203, 745 (1951).
749 G. Medes and N F Floyd, *Biochem. J.* **36**, 259 (1942)
750 E. Borek and H Waelsch, *J Biol. Chem* **141**, 99 (1941).
751 C. Fromageot and P Desnuelle, *Compt rend* **214**, 647 (1942); *Bull. soc. chim biol* **24**, 2169 (1942)
752 R E. Kallio, *J Biol Chem.* **192**, 371 (1951)
753 H. Tarver and C L A Schmidt, *J. Biol Chem* **130**, 67 (1939)
754 V du Vigneaud, W G Kilmer, J. R Rachele, and M Cohn, *J. Biol Chem* **155**, 645 (1944)
755 E. Brand, G F Cahill, and R J. Block, *J Biol. Chem* **110**, 399 (1935).
756 E Brand, R J Block, B Kassell, and G. F. Cahill, *Proc. Soc Exptl Biol Med.* **36**, 501 (1936)
757 V du Vigneaud, G. B Brown, and J. P Chandler, *J. Biol. Chem.* **143**, 59 (1942)
758 [illegible] **143**, 559 (1942)
759 [illegible]
760 [illegible]
761 [illegible]
762 [illegible] neaud, *J.*

indicated that serine was the acceptor. The mechanism involves condensation of homocysteine and serine to yield cystathionine. Cystathionine is cleaved to cysteine and α-ketobutyric acid by rat liver preparations in the presence of cyanide.[763] This system also converts DL-homoserine to α-ketobutyrate Although L-homoserine led to keto acid formation, the keto acid formed was not characterized That α-aminobutyric acid may be formed was suggested by the finding of this amino acid in human urine and an increase in its excretion after methionine administration [135] These reactions may be represented as follows:

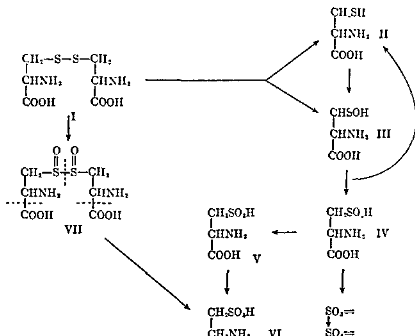


Phosphohomoserine has also been suggested as a product [764] The enzymatic synthesis of cystathionine from homocysteine and serine in a rat liver preparation was recently reported [765] The conversion of N^{15}-labeled serine to N^{15}-labeled cystine was demonstrated by Stetten [766] The formation of cysteine from the methyl group of methionine and the carbon atoms of glycine was shown by Stekol *et al* [767] (see Section V 2) In *Neurospora*[768,770] cystathionine is apparently an intermediate in methionine synthesis from cysteine, and there is evidence that homoserine may be an intermediate in

[763] W R Carroll, G W Stacy, and V du Vigneaud, *J Biol Chem* **180**, 375 (1949).
[764] F Binkley, *J Biol Chem* **155**, 39 (1944)
[765] F Binkley, *J Biol Chem* **191**, 531 (1951)
[766] Stetten D, *J Biol Chem* **144**, 501 (1942)
[767] J A Stekol K Weiss, and S Weiss, *J Biol Chem* **185**, 271 (1950)
[768] H J Teas N H Horowitz, and M Fling, *J Biol Chem* **172**, 651 (1948)
[769] N H Horowitz, *J Biol Chem* **171**, 255 (1947)
[770] M Fling and N H Horowitz, *J Biol Chem* **190**, 277 (1951)

cystathionine formation The conversion of methionine labeled with S^{35} to urinary cystine was demonstrated in a patient with cystinuria [771]

Cystine (I) may be converted to cysteine (II) or cysteine sulfenic acid (III) [772, 773] The oxidation of cysteine to cysteine sulfenic acid is followed by a dismutation leading to the formation of 1 molecule each of cysteine and cysteine sulfinic acid (IV). The latter is oxidized to cysteic acid (V), which may be decarboxylated to taurine (VI). The sulfur of cysteine sulfinic acid may also be oxidized to sulfate. Cystine may be oxidized to cystine disulfoxide (VII), which forms 2 molecules of taurine. These reactions may be formulated as follows·



Cysteine desulfurase, found in certain bacteria[774] and in animal liver, kidney, and pancreas,[775] converts cysteine to hydrogen sulfide, ammonia, and pyruvate The fate of the hydrogen sulfide is probably sulfate Possible intermediates in this conversion may be thiosulfate (which is known to occur in the urine of animals) or free sulfur. Ingested sulfur leads to urinary sulfate in man [776]

[771] L J Reed, D Cavallini, F Plum, J R Rachele, and V du Vigneaud, *J Biol* ...

.. (1942)

)47)

mologia 9, 198 (1940)

[776] H Greengard ... 132, 83 (1940)

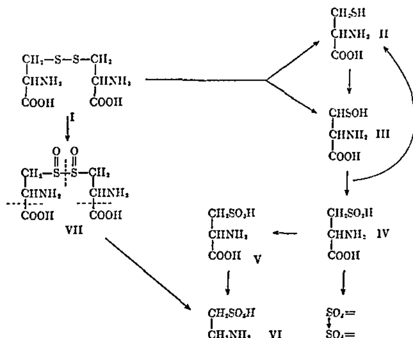
TABLE 7

An Outline of Amino Acid Metabolism

Amino acid	Product of oxidative deamination or transamination	Pathways of metabolism
L-Alanine	Pyruvic acid	
L-Arginine	γ-Keto-δ-guanidovaleric acid	(1) Arginine → Urea + ornithine (2) Arginine → Citrulline + NH_3 (3) Arginine → Agmatine + CO_2
L-Asparagine	α-Ketosuccinamic acid	Asparagine → Aspartic acid + NH_3
L-Aspartic acid	Oxalacetic acid	(1) Aspartic acid ⇌ Fumaric acid + NH_3 (2) Aspartic acid → β-Alanine + CO_2 (3) Aspartic acid → α-Alanine + CO_2
L-Citrulline	α-Keto-δ-carbamido-valeric acid	(1) Citrulline + NH_3 → Arginine (2) Citrulline → Ornithine + CO_2 + NH_3
L-Cysteine	β-Mercaptopyruvic acid	(1) Cysteine → Cysteine sulfinic acid → Cysteic acid → Taurine (2) Cysteine → H_2S + NH_3 + pyruvic acid
L-Glutamic acid	α-Ketoglutaric acid	(1) Glutamic acid → γ-Aminobutyric acid + CO_2 (2) See Histidine, Ornithine, Proline, Glutamine
L-Glutamine	α-Ketoglutaramic acid	Glutamine ⇌ Glutamic acid + NH_3
Glycine	Glyoxylic acid	Glycine + Formate ⇌ Serine
L-Histidine	β-Imidazolepyruvic acid	(1) Histidine → Urocanic acid → Glutamic acid (2) Histidine → Histamine + CO_2
L-Hydroxyproline	α-Keto-γ-hydroxy-δ-aminovaleric acid	
L-Isoleucine	d-α-Keto-β-methylvaleric acid	α-Keto-β-methylvaleric acid → α-Methylbutyric acid → 2-Carbon and 3-carbon fragments
L-Leucine	α-Ketoisocaproic acid	α-Ketoisocaproic acid → Isovaleric acid → Acetoacetic acid
L-Lysine	α-Keto-ε-aminocaproic acid	(1) Lysine → α-Aminoadipic acid → α-Ketoadipic acid → Glutaric acid (2) α-Keto-ε-aminocaproic acid → Dehydropicolinic acid → Pipicolic acid (3) Lysine → Cadaverine + CO_2
L-Methionine	α-Keto-γ-methiolbutyric acid	Methionine → Homocysteine → (1) + Serine → Cystathionine → Cysteine + homoserine (2) H_2S + NH_3 + α-ketobutyric acid
L-Ornithine	α-Keto-δ-aminovaleric acid	(1) Ornithine + NH_3 + CO_2 → Citrulline (2) Ornithine → Putrescine + CO_2
L-Phenylalanine	Phenylpyruvic acid	(1) Phenylalanine → Tyrosine (2) Phenylalanine → Phenylethylamine + CO_2 (3) Phenylpyruvic acid → {Phenylacetic acid / Phenyllactic acid}
L-Proline	α-Keto-δ-aminovaleric acid	(1) Proline → Hydroxyproline (2) Proline → Ornithine (3) Proline → Glutamic acid
L-Serine	β-Hydroxypyruvic acid	(1) Serine → Pyruvic acid + NH_3 (2) Serine + indole → Tryptophan (3) See Glycine
L-Threonine	α-Keto-β-hydroxybutyric acid	Threonine → α-Ketobutyric acid + NH_3
L-Tryptophan	β-Indolepyruvic acid	(1) Tryptophan → Kynurenine → Kynurenic Acid; → Anthranilic acid + alanine; ↓ Nicotinic acid (2) Tryptophan → Tryptamine + CO_2
L-Tyrosine	p-Hydroxyphenylpyruvic acid	(1) p-Hydroxyphenylpyruvic acid → Homogentisic acid → Fumarylacetoacetic acid → Fumaric acid + acetoacetic acid (2) Tyrosine → Tyramine + CO_2
L-Valine	α-Ketoisovaleric acid	α-Ketoisovaleric acid → Isobutyric acid → 3-Carbon fragment + CO_2

cystathionine formation. The conversion of methionine labeled with S^{35} to urinary cystine was demonstrated in a patient with cystinuria.[771]

Cystine (I) may be converted to cysteine (II) or cysteine sulfenic acid (III)[772, 773] The oxidation of cysteine to cysteine sulfenic acid is followed by a dismutation leading to the formation of 1 molecule each of cysteine and cysteine sulfinic acid (IV). The latter is oxidized to cysteic acid (V), which may be decarboxylated to taurine (VI). The sulfur of cysteine sulfinic acid may also be oxidized to sulfate. Cystine may be oxidized to cystine disulfoxide (VII), which forms 2 molecules of taurine These reactions may be formulated as follows:



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[771] L J Reed, D Cavallini, F Plum, J R. Rachele, and V du Vigneaud, *J. Biol*

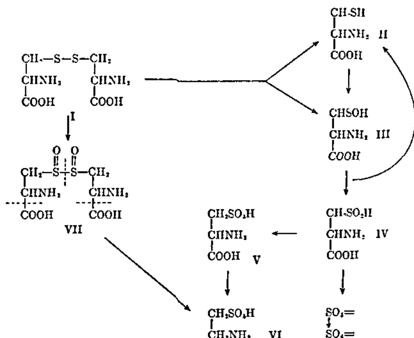
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L-Aspartic acid	Oxalacetic acid	
L-Citrulline	α-Keto-δ-carbamidovaleric acid	
L-Cysteine	β-Mercaptopyruvic acid	Taurine (2) Cysteine → H_2S + NH_3 + pyruvic acid
L-Glutamic acid	α-Ketoglutaric acid	(1) Glutamic acid → γ-Aminobutyric acid + CO_2 (2) See Histidine, Ornithine, Proline, Glutamine
L-Glutamine	α-Ketoglutaramic acid	Glutamine ⇌ Glutamic acid + NH_3
Glycine	Glyoxylic acid	Glycine + Formate ⇌ Serine
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L-Proline	α-Keto-δ-aminovaleric acid	(1) Proline → Hydroxyproline (2) Proline → Ornithine (3) Proline → Glutamic acid
L-Serine	β-Hydroxypyruvic acid	(1) Serine → Pyruvic acid + NH_3 (2) Serine + indole → Tryptophan (3) See Glycine
L-Threonine	α-Keto-β-hydroxybutyric acid	Threonine → α-Ketobutyric acid + NH_3
L-Tryptophan	β-Indolepyruvic acid	(1) Tryptophan → Kynurenine ↗ Kynurenic Acid; ↘ Anthranilic acid + alanine ↓ Nicotinic acid (2) Tryptophan → Tryptamine + CO_2
L-Tyrosine	p-Hydroxyphenylpyruvic acid	(1) p-Hydroxyphenylpyruvic acid → Homogentisic acid → Fumarylacetoacetic acid → Fumaric acid + acetoacetic acid (2) Tyrosine → Tyramine + CO_2
L-Valine	α-Ketoisovaleric acid	α-Ketoisovaleric acid → Isobutyric acid → 3-Carbon fragment + CO_2

cystathionine formation The conversion of methionine labeled with S^{35} to urinary cystine was demonstrated in a patient with cystinuria.[771]

Cystine (I) may be converted to cysteine (II) or cysteine sulfenic acid (III).[772, 773] The oxidation of cysteine to cysteine sulfenic acid is followed by a dismutation leading to the formation of 1 molecule each of cysteine and cysteine sulfinic acid (IV). The latter is oxidized to cysteic acid (V), which may be decarboxylated to taurine (VI). The sulfur of cysteine sulfinic acid may also be oxidized to sulfate. Cystine may be oxidized to cystine disulfoxide (VII), which forms 2 molecules of taurine. These reactions may be formulated as follows:



Cysteine desulfurase, found in certain bacteria[774] and in animal liver, kidney, and pancreas,[775] converts cysteine to hydrogen sulfide, ammonia, and pyruvate The fate of the hydrogen sulfide is probably sulfate Possible intermediates in this conversion may be thiosulfate (which is known to occur in the urine of animals) or free sulfur Ingested sulfur leads to urinary sulfate in man[776]

771 L J Reed, D Cavallini, F Plum, J R. Rachele, and V du Vigneaud, *J Biol* [illegible]

772 .

773

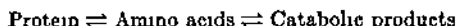
774 .

775 1940)

776

excreted, this process was termed "exogenous" metabolism Although the theory was useful, its underlying concept of the organism as a combustion engine was in error

In the intervening years the need for modification was recognized and an alternative theory was proposed by Borsook and Keighley,[3] which postulated a dynamic steady state of equal breakdown and synthesis of protein on the basis of evidence that was mainly indirect The final overthrow of Folin's theory came with the work of Schoenheimer and his colleagues, using N^{15}-labeled amino acids [4] This work showed that tissue proteins must be regarded, not as inert structural substances, but as labile compounds Even in an adult animal in nitrogen balance, there is a continual breakdown and synthesis



The peptide bonds of the protein chains are, therefore, continually being broken and reconstituted, synthesis of proteins occurs both when amino acids are abundantly supplied in the diet and when the animal is fasting Schoenheimer's evidence was incontrovertible

II. General Considerations

Schoenheimer's work was with amino acids labeled with N^{15} Most of the later work has been done with C^{14} as the label, since the latter radioactive isotope has several advantages over N^{15} Much smaller amounts of C^{14} are required as a label, experiments using only 100 mg fresh tissue and lasting only 1 hr can, therefore, be carried out with C^{14}, whereas with N^{15} the experiments were in a whole animal and lasted for 3 days Since most amino acids in the body are continually and rapidly being deaminized and reaminated,[4] the N^{15} exchanges freely with the non-tracer nitrogen from other amino acids (with lysine[5] and threonine[6] the α-amino group is not reconstituted once deamination has occurred), interpretation of results of such experiments is, therefore, more difficult The very long half-life of C^{14} and the ease of measurement make it preferable to either N^{15} or deuterium in most studies of protein biosynthesis

Before turning to a detailed consideration of experimental results, it might be worth while to consider of what advantage it can be to the organism that its proteins are not fixed structures but exist in a dynamic steady state, especially in view of the fact that energy is required to maintain the steady state so far from the thermodynamic equilibrium (see

[3] H Borsook and G Keighley, *Proc Roy Soc (London)* **B118**, 488–521 (1935)
[4] R Schoenheimer, *The Dynamic State of Body Constituents*, Harvard University Press, Cambridge, Mass , 1942
[5] N Weissman and R Schoenheimer, *J Biol Chem* **140**, 779–795 (1941).
[6] D. F Elliott and A Neuberger, *Biochem J* **46**, 207–210 (1950)

CHAPTER 6

The Biosynthesis of Proteins

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I. Introduction

Intensive work on the problems of protein structure and of protein biosynthesis has been carried out by biochemists for more than half a century. With the use of the new experimental tool of isotope-labeled tracers, certain errors in the older theories have been recognized, especially during the last decade; but the method has not given a solution to any of the problems. This chapter will, therefore, necessarily have to be concerned with the half-answered or unanswered questions that still confront the biochemist The Folin theory of protein metabolism[1, 2] that prevailed until about 1940 postulated that protein synthesis went on to only a slight extent in an adult animal in nitrogen equilibrium The structural proteins of the animal body were thought to be subject to a continual but small breakdown and repair, the protein metabolism involved in these changes was called "endogenous" metabolism Most of the protein in the food, since it was not needed for the endogenous metabolism, was hydrolyzed, oxidized, and

* [illegible] of the authors referred to were aided [illegible]

Society.

[1] O Folin, *Am J Physiol* 13, 117–138 (1905)

[2] O. Folin and W Denis, *J Biol Chem* 11, 87–95 (1912)

oxidation did not decrease the count of the protein. No $C^{14}O_2$ was released from the protein by ninhydrin treatment. All the radioactivity of the protein could be accounted for in the isolated amino acid, with the glycine-incubated reticulocytes, however, 79% was accounted for as glycine and 14% as serine, formed from the glycine. Evidence, using Sanger's dinitrofluorobenzene method of end group determination,[10] indicated that the radioactive amino acids were not incorporated into the proteins in such a way as to have a free amino group.

However, in some cases it has been shown that the labeled amino acid has definitely not been incorporated by a peptide linkage. For example, Melchior and Tarver[11] found that treatment with thioglycolic acid of liver-slice proteins, previously incubated with S^{35}-methionine, released a large fraction of the radioactivity, S^{35}-labeled methionine and the cystine formed from it were evidently bound by linkages other than peptide bonds. Similarly, monoethyleneglycol released some of the S^{35}-labeled ethionine from labeled plasma proteins.[12] A large fraction of the counts in rat liver homogenate protein, previously incubated with labeled glycine, were released by dissolving the protein in dilute alkali and dialyzing or by heating it in trichloroacetic acid.[8, 13, 14]

III. Results of *in Vivo* and *in Vitro* Experiments

The rate of amino acid exchange is different in different tissues. Thus, Schoenheimer's group,[15] after feeding an adult rat labeled L-leucine for 3 days, found that the tissues contained the labeled amino acid in the following relative amounts: serum, 100, intestinal wall, 89, kidney, 82, spleen, 65, liver, 56, heart, 53, testes, 46, muscle, 18, hemoglobin, 17, skin, 11. When a relatively high concentration of the labeled amino acid has been attained *in the proteins of an animal by feeding a labeled amino acid over a period* of time, withdrawal of the labeled amino acid from the diet causes a steady decline in the concentration in the proteins. The tissues that had most rapidly incorporated the amino acids showed the most rapid loss.[16, 17]

Using L-leucine labeled with both N^{15} and deuterium, Schoenheimer *et al.*[15] determined that half the leucine of the liver proteins of a rat is replaced by dietary leucine in approximately 7 days. On the assumption that the

[10] R. R. Porter and F. Sanger, *Biochem. J.* **42**, 287–294 (1948).
[11] J. B. Melchior and H. Tarver, *Arch. Biochem.* **12**, 301–308 (1947).
[12] M. Levine and H. Tarver, *J. Biol. Chem.* **192**, 835–850 (1951).
[13] T. Winnick, F. Friedberg, and D. M. Greenberg, *J. Biol. Chem.* **175**, 117–126 (1948).
[14] T. Winnick, *Arch. Biochem.* **27**, 65–74 (1950).
[15] R. Schoenheimer, S. Ratner, and D. Rittenberg, *J. Biol. Chem.* **130**, 703–732 (1939).
[16] D. Shemin and D. Rittenberg, *J. Biol. Chem.* **153**, 401–421 (1944).
[17] I. A. Abdou and H. Tarver, *J. Biol. Chem.* **190**, 781–790 (1951).

Section IV). Any answer can, necessarily, be only speculative A cell must respond to a wide variety of varying conditions, such as growth, reproduction, changing food supply, infection, the production of antibodies and of enzymes, and hypertrophy in response to increased work. Its ability to adapt to such changing circumstances might possibly be easier if its proteins are labile rather than rigid and immutable structures.

One major assumption underlies nearly all the work with tracers This assumption is that the presence of radioactivity in a protein after incubation with a radioactive amino acid or of N^{15} in an amino acid obtained from hydrolysis of a protein after incubation with an N^{15}-labeled amino acid, that either of these findings indicates that the amino acid has been incorporated into the protein by peptide bonds. This assumption has actually been rigorously shown to be true in only one case,[7] in all other work, evidence, more or less incomplete, pointing to the correctness of the assumption has been advanced. Rigorous proof would involve isolation of a radioactive amino acid from a peptide obtained by partial hydrolysis of the radioactive protein. This procedure was carried out in the one case cited above,[7] in which radioactive aspartic acid was isolated from a hexapeptide obtained by partial enzymatic hydrolysis of egg albumin. Somewhat less rigorous proof was obtained by Winnick *et al*[8] They made use of the well-known fact that ninhydrin liberates carbon dioxide from the carboxyl group of an amino acid when both the carboxyl and amino groups are free; hence, if a carboxyl-C^{14}-labeled amino acid is adsorbed on a protein, or bound in some way other than through its amino or carboxyl group, the carbon dioxide liberated will be radioactive Winnick *et al.* showed that treatment with ninhydrin of rat liver homogenate proteins, previously incubated with carboxyl-C^{14}-labeled glycine, yielded no radioactive carbon dioxide After complete acid hydrolysis, all the C^{14} was released as $C^{14}O_2$; 75% was released after digestion of the protein with a mixture of trypsin, chymotrypsin, carboxypeptidase, and erepsin.

Proteins have been submitted to a variety of treatments to gather evidence that the labeled amino acid is not adsorbed, but that it is chemically bound in the protein. Borsook *et al*,[9] for example, worked with proteins obtained from rabbit reticulocytes after incubation with glycine, L-histidine, L-leucine, or L-lysine, each labeled with C^{14} in the carboxyl group, all their results were in agreement with the interpretation that the labeled amino acids were incorporated by peptide linkages Boiling with trichloroacetic acid, dialysis against an alkaline solution, or performic acid

[7] C B Anfinsen and D. Steinberg, *J Biol Chem* **189**, 739–744 (1951)

[8] T. Winnick, E A. Peterson, and D M Greenberg, *Arch Biochem.* **21**, 235–237 (1949).

[9] H. Borsook, C L Deasy, A J Haagen-Smit, G Keighley, and P. H. Lowy, *J. Biol Chem* **196**, 669–694 (1952)

probably lower than the actual rate for a 1-hr incubation would be. Despite these considerations, the results in Table 2 give the order of magnitude of the rates of *in vitro* incorporations

Some data have been published which could not be included in Table 2 because no estimates of rates of incorporation could be made For example, it has been found that rabbit bone marrow cells convert labeled acetate to aspartic and glutamic acids, which are incorporated into the proteins [34] Phenylalanine is incorporated into the proteins of rat liver homogenate,[35] glycine into intestinal tissue proteins,[36] methionine into an homogenate of rat liver proteins,[25] and serine, after its formation from glycine, into rabbit reticulocyte proteins[9] and into an homogenate of rat liver proteins [8] Liver homogenates from folic acid-deficient rats incorporate glycine more slowly than homogenates from normal rats [27]

In general, it can be concluded that the rate of incorporation into intact cells *in vitro* is of the same order of magnitude as into the corresponding cells *in vivo* Cells that have been damaged by slicing or homogenizing incorporate at a slower rate than intact cells When rabbit bone marrow cells[25] or reticulocytes[9] are lysed, they no longer incorporate amino acids

There is some evidence, from both *in vivo* and *in vitro* work, that the different parts of a single cell do not incorporate a labeled amino acid at the same rates Attempts have been made to correlate the rate of amino

[20] T Peters, Jr and C B Anfinsen, *J Biol Chem* **182**, 171–179 (1950)
[21] T Peters, Jr and C B Anfinsen, *J Biol Chem* **186**, 805–813 (1950)
[22] C B Anfinsen, A Beloff, A B Hastings, and A K Solomon, *J Biol Chem* **168**, 771–772 (1947)
[23] P C Zamecnik, I D Frantz, Jr, R B Loftfield, and M L Stephenson, *J Biol Chem* **175**, 299–314 (1948)
[24] I D Frantz, Jr, R B Loftfield, and W W Miller, *Science* **106**, 544–545 (1947)
[25] H Borsook, C L Deasy, A J Haagen-Smit, G Keighley, and P H Lowy, *J Biol Chem* **186**, 297–307 (1950)
[26] H Borsook, C L Deasy, A J Haagen Smit, G Keighley, and P H Lowy, *J Biol Chem* **186**, 309–315 (1950)
[27] J R Totter, B Kelley, P L Day, and R R Edwards, *J Biol Chem*, **186**, 145–151 (1950)
[28] H Borsook, C L Deasy, A J Haagen Smit, G Keighley, and P J Lowy, *J Biol Chem* **184**, 529–543 (1950)
[29] D Shemin, I M London, and D Rittenberg, *J Biol Chem* **183**, 757–765 (1950)
[30] J B Melchior, M Mellody, and I M Klotz, *J Biol Chem* **174**, 81–88 (1948)
[31] J B Melchior, O Klioze, and I M Klotz, *J Biol Chem* **189**, 411–420 (1951)
[32] R Rutman, E Dempster, and H Tarver, *J Biol Chem* **177**, 491–492 (1949)
[33] J Melchior and H Tarver, *Arch Biochem* **12**, 309–315 (1947)
[34] R Abrams, J M Goldinger, and E S G Barron, *Biochim et Biophys Acta* **5**, 74–80 (1950)
[35] E A Peterson, D M Greenberg, and T Winnick, *Federation Proc* **9**, 214 (1950)
[36] T Winnick, F Friedberg, and D M Greenberg, *Arch Biochem* **15**, 160–161 (1947)

rates of replacement of all the other amino acids are the same, the half-life of rat liver protein is 7 days By following the rate of disappearance of N^{15}-glycine from rat liver protein, *Shemin and Rittenberg*[16] *obtained* a figure for the half-life of 6 days, which is in good agreement with the value found before The average half-life of the total protein in man is 80 days; *in the rat it is 17 days*[18] Sprinson and Rittenberg estimate that turnover in the liver and in the plasma accounts for 41% of the total turnover in man and for 25% in the rat.

Table 1 summarizes results of incorporation of C^{14}-labeled amino acids in *mice over a period of from 10 to 240 min. after intravenous injection.* These injection experiments indicate even more clearly than continuous feeding experiments the relatively great speed with which the visceral

TABLE 1

AMOUNTS OF LABELED AMINO ACIDS FOUND IN VISCERAL AND PLASMA PROTEINS OF MICE AT SUCCESSIVE TIME INTERVALS AFTER INTRAVENOUS INJECTION[19]
(Results expressed as micromoles per gram of protein)

Time after injection min	Glycine		Histidine		Leucine		Lysine	
	Viscera	Plasma	Viscera	Plasma	Viscera	Plasma	Viscera	Plasma
10	0 53	0 07	0 66	0 11	1 4	0.36	1 1	0
20	1.4	0 30	1 3	0 65	3 0	0 15	1.7	0 04
30	2 6	1 23	2 9	1 52	3 6	1.33	1 7	0 25
60	2 5	1 31	3 1	3 10	3.6	4 23	1 9	1 11
120	2 2	1 69	2 8	4 37	4 0	4 21	2 0	1 25
240	2 4	2 18	3 2	5 00	4 0	5 47	1.6	0 95

proteins incorporate circulating amino acids into their proteins. Redistribution of the amino acid then follows, as, for example, by loss from the visceral proteins and incorporation into the plasma proteins

In addition to the *in vivo* work just summarized, much work has been done on *in vitro* experiments *in an effort to determine the mechanism of* the incorporation process Table 2 summarizes results of *in vitro* experiments, in which intact tissues, cells, slices, homogenates, or homogenate fractions were used The literature values have been recalculated to give the results in terms of micromoles of labeled amino acid incorporated per gram of protein per hour Since details required for the calculation were not given in some of the original papers, the values in these cases are only *estimates The calculated results are average rates* for the incubation period, and, since the rate of incorporation usually decreases, are therefore

[18] [illegible] (1949)
[19] [illegible] Lowy.

acid turnover with the function of various cellular constituents Results of *in vivo* incorporation of four labeled amino acids into four fractions of guinea pig liver are given in Table 3 The microsome fraction showed the most rapid rate of incorporation of all four amino acids. As would be expected, the loss of nitrogen during protein starvation is also not equally distributed among the cell constituents The greatest loss occurred in the mitochondrial and microsome fractions, with little or no loss in the nuclear fraction

In vitro, it was similarly found that the rates of incorporation were not the same in the various cell constituents (Table 2) Siekevitz and Zamecnik[37] found, using rat liver cell fractions with DL-alanine, that the relative rates of incorporation were microsomes, 15, mitochondria, 6, homogenate, 3, supernatant, 1. Comparing these data with the data in Table 2, it can

TABLE 3

INCORPORATION *in Vivo* OF LABELED AMINO ACIDS INTO INTRACELLULAR FRACTIONS OF GUINEA PIG LIVER 30 MIN AFTER INTRAVENOUS INJECTION OF 1 6 MG PER 100 G BODY WEIGHT[19]

(Results expressed as micromoles per gram of protein)

Fraction	Labeled amino acid			
	Glycine	L-Histidine	L-Leucine	L-Lysine
Nuclear fraction	0 56	1 5	2 3	1 3
Mitochondria	0 60	1 2	1 1	1 6
Microsomes	1 2	3 1	4 3	2 9
Supernatant	0 69	1 2	1 8	1 6

be seen, therefore, that the relative speeds of incorporation of the cell constituents is not the same for every amino acid Particularly interesting is the finding that, in the case of lysine, the optimal conditions for incorporation into one cellular fraction are not the same as the optimum conditions for another[38] Also of interest is the report that, in the incorporation of alanine into rat liver proteins, the mitochondria act synergistically on the incorporation by the microsomes[37] (see also ref. [53])

In view of the fact that mitochondria and microsomes are very active enzymatically, it is not unexpected to find that enzymes also undergo active amino acid turnover For example, when bovine pancreas slices were incubated in saline with $C^{14}O_2$, the ribonuclease subsequently isolated was approximately twice as active as the average of the slice proteins[39] It has also been found that maintenance of rats on low or non-protein diets is

[37] P Siekevitz and P C Zamecnik, *Federation Proc* 10, 246-247 (1951)

[38] H Borsook, C L Deasy, A J Haagen-Smit, G Keighley, and P H Lowy, *J Biol Chem* 179, 689-704 (1949)

[39] C B Anfinsen, *J Biol Chem* 185, 827-831 (1950)

TABLE 2

INCORPORATION OF LABELED AMINO ACIDS INTO TISSUE PROTEINS *in Vitro*

Tissue	Labeled compound	Incubation time, hr	Micromoles per gram of protein per hour	Reference
Liver slices, chicken	$C^{14}O_2$	3.4	1.15-3.3 in slice proteins; 40-256 in serum albumin	20, 21
Liver slices, rabbit	$C^{14}O_2$	3	3.2	22
Liver slices, rat	Alanine	3.5	0.6	23
Liver slices, rat	Alanine	2	0.085	24
Liver hepatoma slices, rat	Alanine	3.5	5.1	23
Liver slices, foetal rat	Alanine	3.5	3.6	23
Liver homogenate, mouse	Alanine	4	0.06	14
Liver homogenate, rat	Alanine	4	0.04	14
Liver homogenate, foetal rat	Alanine	4	0.50	14
Tumor homogenate, mouse	Alanine	4	0.3	14
Bone marrow cells, rabbit	*Glycine*	1	0.5	25
Diaphragm, rat	Glycine	1	0.1	26
Reticulocytes, rabbit, in saline	Glycine	1	0.78	9
Reticulocytes, rabbit, in plasma	Glycine	1	1.29	9
Liver slices, rat	Glycine	3.5	3.0	23
Liver hepatoma slices, rat	Glycine	3.5	8.9	23
Liver, homogenate, chicken	*Glycine*	1-1.5	0.11-0.25	27
Liver, homogenate, rat	Glycine	4	0.08	14
Liver, homogenate, rat	Glycine	2	0.18 as glycine; 0.12 as serine	8
Liver, homogenate, mouse	Glycine	4	0.08	14
Liver, homogenate, foetal rat	Glycine	4	0.55	14
Tumor homogenate, mouse	Glycine	4	0.08	14
Liver "nuclei," guinea pig	Glycine	1	0.125	28
Liver, mitochondria guinea pig	Glycine	1	0.10	28
Liver, microsomes guinea pig	Glycine	1	0.019	28
Blood, duck	*Histidine*	12	0.83	29
Reticulocytes, rabbit, in saline	Histidine	1	1.08	9
Reticulocytes, rabbit, in plasma	Histidine	1	1.75	9
Bone marrow cells, rabbit	Leucine	1	2.9	25
Diaphragm, rat	Leucine	1	0.1	26
Reticulocytes, rabbit, in saline	Leucine	1	0.7	9
Reticulocytes, rabbit, in plasma	Leucine	1	2.6	9
Liver, guinea pig, "nuclei"	Leucine	1	0.15	28
Bone marrow cells, rabbit	Lysine	1	1.8	25
Diaphragm, rat	Lysine	1	0.1	26
Reticulocytes, rabbit, in saline	Lysine	1	0.99	9
Reticulocytes, rabbit, in plasma	Lysine	1	2.51	9
Liver, "nuclei," guinea pig	Lysine	1	2.1	28
Liver, mitochondria guinea pig	Lysine	1	1.6	28
Liver, microsomes, guinea pig	Lysine	1	0.45	28
Liver, particle free supernatant, guinea pig	Lysine	1	1.6	28
E coli, resting	Methionine	6	0.004-0.11	30, 31
Liver slices, rat	Methionine	2	0.13-0.27 as cystine; 0.19-0.40 as methionine	32, 33

incorporation and initial concentration may explain in part the finding that a thirtyfold increase in the amount of glycine injected only doubled its rate of incorporation into the liver [54]

Two labeled amino acids that are not normal constituents of animal proteins have been tested *in vivo* to determine whether unnatural amino acids would be incorporated α-Aminoadipic acid, when injected into mice, was not incorporated into the proteins, although it was catobalized at approximately the same rate as lysine [19] Ethionine, however, is incorporated into rat tissue proteins [55] Although the degree of incorporation was only approximately one-tenth of that of normal amino acids, and the half-life was less, nevertheless the finding that an unnatural amino acid can be incorporated into a protein is an important one In the one case studied, an amino acid of the D configuration, D-lysine, was not incorporated [38]

In most *in vitro* chemical reactions the products are formed in amounts large enough to be measured by the ordinary methods This is not the case in protein synthesis, since the reaction is very slow. Furthermore, any small increase in protein can be seen only against the background of the relatively large amount of protein necessarily present as the enzyme system In one case, however, a net synthesis of protein has been demonstrated [20, 21] Peters and Anfinsen incubated chicken liver slices with $C^{14}O_2$ and found that 0 42 mg of protein was synthesized per gram of liver (wet weight) in 4 hr The synthesized protein has the physical, chemical, and immunological properties of serum albumin In the total serum albumin at the end of the experiment, 37 5% of the carboxyl groups of the dicarboxylic amino acids was labeled

Of general biological interest is the conclusion, which must be drawn from the *in vitro* experiments with individual liver cell fractions, that incorporation of amino acids into proteins does not, in the adult cell, necessarily depend on direct participation of the nucleus The explanation that could be advanced for the *in vivo* findings, that the incorporation occurred first in the nucleus, the labeled protein later being transferred to the extranuclear particles and to the particle-free cytoplasm, is excluded in the *in vitro* work

There is a considerable body of indirect evidence that relates nucleic acid to protein synthesis A number of workers[56-65] associate ribosenucleic

[54] D M Greenberg and T Winnick, *J Biol Chem* **173**, 199–204 (1948)

[55] M Levine and H Tarver, *J Biol Chem* **192**, 835–850 (1951)

[56] W C Schneider, *Cold Spring Harbor Symposia Quant Biol* **12**, 169–178 (1947)

[57] J Brachet, *Arch biol (Paris)* **44**, 519–576 (1933)

[58] T Caspersson, H Landström-Hydén, and L Aquilonius, *Chromosoma* **2**, 111–131 (1941–1944)

[59] H Hydén, *Acta Physiol Scand* **6**, *Suppl.* **17**, 1–136 (1943)

[60] S Spiegelman and M D Kamen, *Cold Spring Harbor Symposia Quant Biol* **12**, 211–223 (1947)

associated with loss in the liver of catalase, alkaline phosphatase, xanthine dehydrogenase, cathespin, and arginase; a shift to a high protein diet led to prompt restoration of enzyme activity and of total protein.[40]

Tumors *in vivo* incorporate labeled amino acids approximately as fast as such active tissues as liver and kidney.[16, 41-50] Although Shemin and Rittenberg found that transplantable sarcoma R-39 releases N^{15}-labeled glycine from its proteins at about one-quarter the rate of liver,[16] in all other cases reported the release of the labeled amino acid from tumor tissue was at about the same rate as from liver and kidney.

In general, tumor slices or homogenates incorporate labeled amino acids faster than the corresponding normal tissues (Table 2). As might be expected, foetal tissues *in vitro* incorporate labeled amino acids faster than adult tissues (Table 2) [51] Regenerating adult liver also incorporates labeled amino acids faster than normal adult liver [52]

The rate of *in vitro* incorporation of labeled amino acids is a logarithmic function of its initial concentration up to a certain optimum concentration; concentrations above 0 001 to 0 003 *M* were inhibitory. One exception to this rule has been noted: the rate of incorporation of lysine into liver homogenate (but not into other tissues) is a linear function of the initial concentration of the amino acid.[53] This, however, is an exceptional case in a number of other respects (see p 201) In general, tissues incorporate *in vitro* amino acids at concentrations similar to those in the blood, and the dependence of the rate of incorporation on concentration is greatest in the physiological range [25, 26 33, 53] The logarithmic relationship between rate of

[40] L L Miller, *J Biol Chem* **186**, 253–260 (1950)

[41] T Winnick, F Friedberg, and D M Greenberg, *J Biol Chem* **173**, 189–197, (1948)

[42] A C Griffin, S Bloom, L Cunningham, J D Teresi, and J M Luck, *Cancer* **3**, 316–320 (1950)

[43] G A LePage and C Heidelberger, *Federation Proc* **9**, 195 (1950)

[44] G A LePage and C Heidelberger, *J Biol Chem* , **188**, 593–602, (1951)

[45] A J. Kemen, S W Hunter, G E Moore, and C. R Hitchcock, *Cancer Research* **9**, 174–176 (1949)

[46] Reid, J C and Jones, H B , *J Biol Chem* **174**, 427–437 (1948)

[47] I. D Frantz, Jr , P C Zamecnik, J W Reese, and M L Stephenson, *J Biol Chem* **174**, 773–774 (1948)

[48] P C Zamecnik and I D Frantz, Jr , *Cold Spring Harbor Symposia Quant Biol* **14**, 199–208 (1949)

[49]

[50] 9, 262 (1951)

[51] *m* **173**, 437–438 (1948)

[52] D M Greenberg, F Friedberg, M P Schulman, and T Winnick, *Cold Spring Harbor Symposia Quant Biol* **13**, 113–117 (1948)

[53] H Borsook, C L Deasy, A J Haagen Smit, G Keighley, and P H Lowy, *Federation Proc* **8**, 589–596 (1949)

some evidence that in the alloxan diabetic rat there is decreased synthesis, rather than increased catabolism [80] This conclusion is supported by studies[81] on the rates of incorporation of S^{35}-labeled methionine into skeletal muscle proteins of normal and depancreatized dogs Five hours after injection of the labeled amino acids, i e , at a time when the loss of labeled amino acid previously incorporated is negligible, there was twice as much incorporation in the muscle protein of the normal animal. Injection of insulin increased incorporation in the diabetic dog three- or fourfold

IV. *Energy Considerations in Protein Synthesis*

The formation of a peptide from two amino acids is accompanied by an increase in free energy, in other words, peptide formation from free amino

TABLE 4

FREE ENERGIES AND EQUILIBRIUM CONSTANTS OF FORMATION OF SOME PEPTIDE BONDS (CALORIES PER MOLE)[82, 83]

Reaction	Pure Substance $-\Delta F_{298.1}$	Aqueous Solution $-\Delta F_{298.1}$	Aqueous Solution $K_{298.1}$
DL-Alanine + glycine → DL-Alanylglycine + water	−3560	−4230	0 00106
2 Glycine → Glycylglycine + water	−2440	−5905	0 00299
Benzoate ion + glycine → Hippurate ion + water		−2630	0 01415
Benzoic acid + glycine → Hippuric acid + water	−2220		
Hippuric acid + glycine → Hippurylglycine + water	−2170		
DL-Leucine + glycine → DL-Leucylglycine + water	−2850	−3416	0 00252

acids does not proceed spontaneously to any significant extent At equilibrium, the peptide is nearly completely hydrolyzed

In order to show the order of magnitude of the energy changes involved, the free energies of formation of some peptide bonds, together with the equilibrium constants of the reactions, are given in Table 4 The data show that the free energy of formation of a peptide is greater in solution than in the pure state In the tissues, where the concentrations are of the order of magnitude of 0 001 *M* or less, the amount of the above peptides present at equilibrium would be infinitesimal

[80] H D Hoberman, *J Biol Chem* **188**, 797–804 (1951)

[81] L L Forker, I L Chaikoff, C Entenman, and H Tarver, *J Biol Chem* **188**, 37–48 (1951)

[82] H Borsook, unpublished data

[83] H Borsook and J W Dubnoff, *J Biol Chem* **132**, 307–324 (1940)

acid with cytoplasmic protein synthesis, although desoxyribosenucleic acid, confined to the nucleus, is not excluded from the process Notable in this connection is the fact that microsomes, which of all the cell constituents have the highest concentration of ribosenucleic acid,[58] have the most rapid *in vivo* incorporation.[19, 66] In the development of reticulocytosis in rabbits, there is a thirty- to fortyfold increase in ribosenucleic acid and a parallel increase in ability to incorporate labeled amino acids.[67] The increase in desoxyribosenucleic acid is only two- to threefold

That *protein synthesis is affected by hormones has been known for a long* time Thus, not only the growth hormone[68] but also the pituitary and steroid sex hormones[69-76] are known to affect the balance between synthesis and breakdown of protein; loss of weight is a salient feature of diabetes *and of hyperthyroidism. Experiments on hormonal effects, using* labeled amino acids, are difficult to interpret, since the final result may be due to changes in the rates both of incorporation and of breakdown Friedberg and Greenberg[77] found that injection of anterior pituitary growth hormone caused a 70% greater incorporation of S^{35}-labeled methionine into proteins

rats[78] Taking a decrease of amino acids in the blood as evidence of protein synthesis and an increase as evidence of breakdown, Lotspeich[79] found that carbohydrate promotes synthesis in the normal animal and breakdown *in* the diabetic; insulin promotes synthesis in the diabetic animal. There is

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62 J Brachet, *Cold Spring Harbor Symposia Quant Biol* 12, 18–27 (1947)
63 [illegible] 12, 35–49 (1947)
64 [illegible] 0–59 (1947)
65 [illegible] 14 (1947)
66 [illegible] 55 (1947)
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68 [illegible] 952)
69 [illegible] 1947)
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71 [illegible])
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73 [illegible] 182, 581–589 (1950)
74 E C Reifenstein, Jr , F Albright, and S L Wells, *J Clin Endocrinol* 5, 367–395 (1945); correction 6, 232 (1946)
75 C D Kochakian, *Am J Physiol* 145, 549–556 (1946)
76 C D. Kochakian, J H Humm, and M N Bartlett, *Am J Physiol* 155, 242–250 (1948)
77 F. Friedberg and D M. Greenberg, *Arch Biochem.* 17, 193–195 (1948)
78 F Friedberg, *Euclides* 109, 116–118 (1950)
79 W D Lotspeich, *J. Biol Chem* 185, 221

incorporated by both methods A direct experimental method of attack on this problem has not yet been found

Is the mechanism of incorporation in any one tissue the same for all amino acids? Is the mechanism of incorporation for any one amino acid the the same in every tissue? From one series of *in vitro* experiments,[33] it would appear that lysine is incorporated into the proteins of guinea pig liver homogenate by two different mechanisms The enzyme for incorporation by one mechanism is confined to the particle-free supernatant, its optimum pH is in the neighborhood of 6 5, and it requires added calcium ions The enzyme for the second type of incorporation is chiefly in the mitochondria, its optimum pH is near 7 5, and it does not require added calcium ions The enzymes for the two types of reaction also differ in their behavior toward inhibitors, arsenite (0 001 *M*), for example, inhibits the first reaction 67 %, but it has no effect on the second reaction

However, there is evidence, from the effects of various inhibitors, that the mechanism of incorporation of some amino acids in some tissues is similar Thus, in rabbit bone marrow cells[25] the incorporation of glycine, of leucine, and of lysine was inhibited completely by anaerobiosis, arsenite, and 2,4-dinitrophenol Arsenate inhibited the incorporation of glycine, leucine, and lysine 96, 77, and 80%, respectively, azide, 84, 77, and 68% In rat diaphragm,[26] anaerobiosis, arsenite, and 2,4-dinitrophenol again inhibited completely the incorporation of the same three amino acids Arsenate inhibited their incorporation 67, 58, and 63%, respectively, azide, 85, 78, and 85% With rabbit reticulocytes,[9] the percentage inhibitions of the incorporation of glycine, histidine, leucine, and lysine, respectively, were. anaerobiosis, never complete but very variable, arsenite, 90, 95, 96, 97, 2,4-dinitrophenol, 93, 83, 81, 86, arsenate, 29, 18, 20, 46, azide, 30, 19, 13, 30, diethyldithiocarbamate, 19, 19, 37, 53, α,α'-dipyridyl, 64, 46, 62, 69, fluoride (0 02 *M*), 99, 97, 99, 100, fluoride (0 001 *M*), 0, 0, 1, 0, hydroxylamine (01 *M*), 89, 89, 93, 95, hydroxylamine (001 *M*), 0, 0, 0, 0, iodoacetate, 88, 53, 51, 79, ammonium molybdate, 42, 55, 61, 33 The similarity of the degree of inhibition with the four amino acids in the three tissues is a strong indication that the incorporations proceeded by the same mechanism

If the incorporation of labeled amino acids proceeds by the first of the mechanisms postulated by Schoenheimer, i e , by complete breakdown of the protein into its units followed by resynthesis, one would expect that the presence of all other amino acids contained in the protein would be necessary for the incorporation of the labeled amino acid On the other hand, if the incorporation proceeded by the second mechanism, the presence of the other amino acids should not be obligatory The experimental evidence unfortunately, is not sufficient to make a definite decision possible

The heat evolved in the early stages of peptic digestion of β-lactoglobulin, when the products are almost entirely peptides of varying size, is 1300 cal. per mole of peptide bond split[34] The heat of formation of a dipeptide is in the neighborhood of −3000 to −4000 cal[35] Since ΔH and ΔF in these reactions are nearly of the same order of magnitude, it would appear that the greatest energy requirement in the synthesis of proteins would be in the formation of small peptides from the free amino acids Once the smaller peptides are formed, the synthesis of a protein molecule from the peptides (if this be the mechanism by which proteins are formed) would require a relatively small amount of energy

It also should be noted (Table 3) that the free energy change in the *condensation of two amino acids is significantly greater than the free energy* change in the formation of hippuric acid from benzoic acid and glycine Some reliable general conclusions may be obtainable from study of more models of this and other types, particularly where the positive and negative changes on the reactants are different distances apart or where one change is absent

In spite of the fact that the data indicate that the equilibrium position corresponds to over 99% hydrolysis, the view that peptide and protein synthesis can be catalyzed by peptidases and proteases has been advanced The free energy data show unequivocally that, whether or not this is the case, the reaction cannot, under physiological conditions, be a simple mass *action reversal of hydrolysis*

V. Mechanisms of Peptide Bond Synthesis

It should be emphasized that, in all cases but one (see p. 197), experi*ments with labeled amino acids do not differentiate between synthesis of* protein and amino acid turnover, i.e , replacement of one amino acid residue by another amino acid residue of the same structure Whether the second process occurs at all is uncertain Certainly the energy requirements for synthesis of a peptide bond would be of an entirely different order of magnitude than the energy requirement for mere replacement of one amino acid combined in peptide linkage by another.

If the reaction is simply replacement, it could occur by two different possible mechanisms as first postulated by Schoenheimer *et al* .[15] (1) complete breakdown of the protein into its units, followed by resynthesis, or (2) only partial replacement of units, by an enzyme-mediated exchange of amino acids, without hydrolysis of the moieties on either side of the two peptide bonds split in the interchange Or, possibly, amino acids may be

[34] G Haugaard and R M Roberts, *J Am Chem Soc* 64, 2664–2671, (1942)

[35] H Borsook and H M Huffman, *in* Schmidt, Chemistry of the Amino acids and *Proteins*, *Charles* C Thomas, Springfield, Ill 1938, pp 822–870

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Some evidence in favor of the first mechanism has come from feeding experiments. An animal is fed a diet lacking in one of the indispensable amino acids, with the missing indispensable amino acid being fed or injected separately.[86-96] If the animal receives the lacking indispensable amino acid more than a few hours later than the main dietary mixture, the supplemented diet is ineffective for growth, or for recovery from protein depletion, or for maintenance. The interpretation placed on this finding has been that all the amino acids must be present at concentrations greater than the fasting levels for protein synthesis to occur. In agreement with this interpretation is the observation that young rats on a tryptophan-deficient diet incorporated intraperitoneally injected C^{14}-labeled tryptophan more slowly than litter mates receiving tryptophan in their diets.[97]

On the other hand, it has been found that the rate of incorporation of C^{14}-labeled tryptophan and glycine is only slightly reduced in animals on a phenylalanine-deficient diet.[97] However, this decreased rate of incorporation may be due simply to the fact that animals on deficient diets eat less and have, therefore, a decreased total protein turnover.

More evidence in favor of the second Schoenheimer mechanism comes from the fact that a labeled amino acid, whether dispensable or indispensable, is incorporated very rapidly after injection, both in an animal on a normal diet and in a fasting animal.[98]

In vitro experiments, in which the rate of amino acid incorporation in the presence of other amino acids was measured, also indicate that, in general, the second mechanism is the correct one. For example, labeled glycine, leucine, and lysine, when incubated as a mixture with guinea pig liver mitochondria, rat diaphragm, or rabbit bone marrow cells, give a protein with a count that is the sum of the counts of the protein obtained from incubations with the amino acids individually.[25, 26, 53] In other words, one could conclude that each amino acid is incorporated independently of the others. Addition of a mixture of amino acids approximating the

86 [illegible] (1947)
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88 300–311 (1947)
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90 315–320 (1949)
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93 [illegible] R C Stepto, *Federation Proc.* **6**, 390 (1947)
94 K M. Yeshoda and M Damodaran, *Biochem J*, **41**, 382–388 (1947)
95 R A Harte, J J Travers, and P Sarich, *J Nutrition* **35**, 287–293 (1948)
96 R Henderson and R A Harris, *Federation Proc* **8**, 385 (1948)
97 D. R. Sanadi and D M Greenberg, *Proc Soc Exptl Biol Med* **69**, 162–163 (1948)
98 H. Tarver and C L A Schmidt, *J Biol Chem* **146**, 69–84 (1942)

composition of casein or of hemoglobin (with omission of the amino acid corresponding to the labeled amino acid) did not affect the uptake of labeled glycine, leucine, or lysine in the systems On the other hand, in experiments in which rabbit reticulocytes are incubated in saline or in plasma with labeled glycine, histidine, leucine, or lysine, there is evidence that the incorporation of one amino acid is not necessarily independent of the presence of all other amino acids [9] The incorporation of these labeled amino acids into reticulocytes is accelerated, in saline, by histidine, phenylalanine, and valine, and, in plasma, also by leucine None of the other amino acids had any effect Only histidine had an accelerating effect when added alone, leucine, phenylalanine, and valine required histidine to exert their accelerating effects

In all *in vitro* experiments it should be noted that the tissue always contains some free amino acids which may be sufficient in amount to invalidate conclusions based on the assumption that only the added amino acids are present As matters now stand, the experimental evidence cannot be interpreted as favoring either mechanism

VI. Possible Intermediates in Protein Synthesis

In the section on energy considerations in protein synthesis, it has been shown that the formation of a peptide bond represents an increase in free energy and that synthesis of a protein molecule probably does not occur by a simple reversal of hydrolysis If this statement is true, then it would appear probable that the formation of some energy-rich intermediate is the first requirement for peptide bond formation Accordingly, there has been a continual search for such possible intermediates

The first intermediates to be considered are peptides As has been previously stated, the energy requirements for the condensation of peptides are probably less than those for the condensation of free amino acids The phenomenon of plastein formation is in support of this idea, when an enzymatic hydrolyzate of a protein is concentrated, with added pepsin or chymotrypsin products are obtained which have molecular weights in the range of 250,000 to 400,000 as determined in the ultracentrifuge [99, 114] As

[99] H Wasteneys and H Borsook, *Physiol Revs* **10**, 110–145 (1930)
[100] H Borsook, *Physiol Rev* **30**, 206–219 (1950)
[101] S J Folley, *Biochem J* **27**, 151–152 (1933)
[102] P G E Ecker, *J Gen Physiol* **30**, 399 (1946–1947)
[103] W T Salter and O H Pearson, *J Biol Chem* **112**, 579–589 (1935–1936)
[104] H B Collier, *Canadian J Res* **B18**, 255–263 (1940)
[105] H. B Collier, *Canadian J Res* **B18**, 272–280 (1940)
[106] J H Northrop, *J Gen Physiol* **30**, 377–378 (1946–1947)
[107] A I Virtanen and H K Kerkkonen, *Acta Chem Scand* **1**, 140–141 (1947)
[108] A I Virtanen and H K Kerkkonen, *Nature* **161**, 888–889 (1948)

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87 E Geiger, *J Nutrition* **36**, 813–819 (1948)
88 A J Schaeffer and E Geiger, *Proc Soc Exptl Biol Med.* **66**, 309–311 (1947)
89 E Geiger, *Science* **108**, 42–43 (1948)
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93 *Federation Proc* **6**, 390 (1947)
94 K M Yeshoda and M Damodaran, *Biochem J*, **41**, 382–388 (1947)
95 R A Harte, J J Travers, and P Sarich, *J Nutrition* **35**, 287–293 (1948)
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[102] P. G. E. Ecker, *J. Gen. Physiol.* **30**, 399 (1946–1947).
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[104] H. B. Collier, *Canadian J. Res.* **B18**, 255–263 (1940).
[105] H. B. Collier, *Canadian J. Res.* **B18**, 272–280 (1940).
[106] J. H. Northrop, *J. Gen. Physiol.* **30**, 377–378 (1946–1947).
[107] A. I. Virtanen and H. K. Kerkkonen, *Acta Chem. Scand.* **1**, 140–141 (1947).
[108] A. I. Virtanen and H. K. Kerkkonen, *Nature* **161**, 888–889 (1948).

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[87] E Geiger, *J Nutrition* 36, 813–819 (1948)
[88] A J Schaeffer and E Geiger, *Proc Soc. Exptl Biol Med* 66, 309–311 (1947)
[89] E Geiger, *Science* 108, 42–43 (1948)
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[93] *Federation Proc* 6, 390 (1947)
[94]
[95] (1948)
[96] 9, 162–163
[97] (1948)
[98] H Tarver and C L A Schmidt, *J Biol Chem* 146, 69–84 (1942)

phorylated amino acid derivative Here again, however, a labeled phosphorylated derivative has not been isolated in experiments in which labeled amino acids were incorporated There is some indirect evidence from model experiments that phosphorylated derivatives are not intermediates For example, Cohen and McGilvery[118] found that, although ATP promoted the enzymatic synthesis of *p*-aminohippuric acid from *p*-aminobenzoic acid and glycine under anaerobic conditions, N-phosphorylated glycine was inactive Similarly, N-phosphorylated glycine and benzoylphosphate were found to be no more active than glycine and benzoic acid in the enzymatic synthesis of hippuric acid [119] Chantrenne[120] also demonstrated that benzoylphosphate is unable to replace a mixture of benzoic acid and ATP in the enzymatic synthesis of hippuric acid, a fact which excludes free benzoylphosphate as an intermediate in the reaction

Dehydropeptides are excluded as possible intermediates in the synthesis of protein by *E coli*,[117] since glycyldehydrophenylalanine did not support growth in a mutant requiring L-phenylalanine, whereas the corresponding normal peptide, glycylphenylalanine, did support growth

VII. Model Experiments in Peptide Bond Synthesis

Since the problem of peptide bond synthesis is complicated in the case of proteins, which are large molecules of unknown structure, many attempts have been made to study peptide bond synthesis in simpler molecules, i e , in so-called "model" systems There have been two types of systems studied· (1) those in which actual net synthesis of peptide bonds occurred, and (2) those in which there were exchange or transfer reactions, without a net increase of peptide bonds in the system The former type will be considered first

Bergmann has carried out a brilliant series of experiments in which various derivatives of amino acids are condensed enzymatically to form peptide bonds For example, carbobenzoxyglycine anilide was formed in 80% yield from carbobenzoxyglycine and aniline by incubation at 40° and pH 4 6 with activated papain Similarly acetyl, benzoyl, and carbobenzoxy derivatives of alanine, leucine, and phenylalanine gave with aniline and phenylhydrazine the corresponding anilides and phenylhydrazides, acetylphenylalanylglutamic acid and *p*-toluenesulfonylglycine gave with aniline the corresponding anilides However, although hippuric acid condensed with aniline to give hippuric acid anilide, there was no synthesis of the amide from hippuric acid and ammonia [121, 122]

[118] P P Cohen and R W McGilvery, *J Biol Chem* **171**, 121–133 (1947)
[119] H Borsook and J W Dubnoff, unpublished observations
[120] H Chantrenne, *J Biol Chem* **189**, 227–233 (1951)
[121] M Bergmann and H Fraenkel-Conrat, *J. Biol Chem* **119**, 707–720 (1937)
[122] M Bergmann and H Fraenkel-Conrat, *J. Biol Chem* **124**, 1–6 (1938)

would be expected from the energy considerations, no plastein formation occurs when the initial reaction mixture consists of either free amino acids or di- and tripeptides; with peptides of greater complexity, plastein formation takes place.

Indirect evidence that might lead to the conclusion that peptides are intermediates has been obtained in one case. Anfinsen and Steinberg[7] digested the ovalbumin obtained by incubating minced hen's oviduct with $C^{14}O_2$ with a bacterial enzyme and isolated the aspartic acid from a hexapeptide and from the plakalbumin thus obtained. The specific activity of the aspartic acid in the hexapeptide was always greater than that in the plakalbumin, the ratio varying from 1.3 to 3 5 Anfinsen and Steinberg concluded that this finding suggests that peptides are intermediates in the synthesis, since, if the synthesis (or incorporation) had occurred directly from the pool of free amino acids, the specific activities should have been the same for all parts of the ovalbumin molecule. However, there is another possible explanation of the data: there was a direct interchange of free aspartic acid with the aspartic acid in the ovalbumin, but the interchange occurred at different rates in different parts of the molecule, the rate being affected by the amino acids with which the aspartic acid was linked.

Another possible intermediate suggested for protein synthesis is an N-acetylated amino acid, the assumption is made that the amino group would be sufficiently activated to condense with the carboxyl group of another amino acid [115-116] The positive evidence in support of this theory is the fact that animals are known to acetylate unnatural amino acids and amines. However, a labeled N-acetylated amino acid has not been isolated in experiments in which labeled amino acids were incorporated, and, in one model experiment in which *p*-aminohippuric acid was enzymatically synthesized from *p*-aminobenzoic acid and glycine, it was found that N-acetylglycine was inactive [116] N-Acetylated amino acids are also excluded as intermediates in protein synthesis in *E. coli*, since acetyl-L-phenylalanine and acetyl-L-tyrosine were unable to support growth in two mutants which required L-phenylalanine and L-tyrosine, respectively.[117]

Another type of possible intermediate that has been suggested is a phos-

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109 A I Virtanen, H K Kerkkonen, T Laaksonen, and M Hakala, *Acta Chem Scand* **3**, 520-524 (1949)

110 A I Virtanen, H K Kerkkonen, M Hakala, and T Laaksonen, *Naturwissenschaften* **37**, 139-140 (1950)

111 H Tauber, *J. Am Chem Soc* **71**, 2952-2953 (1949)

112 H Tauber, *J Am Chem Soc* **73**, 1288-1290 (1951)

113 — — *J Am Chem Soc* **73** 4965-4966 (1951)

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115 **15**, 247-272 (1946)

116 R M Herbst and D [illegible], 541-547 (1943)

117 S Simmonds, E. L Tatum, and J S Fruton, *J Biol Chem* **169**, 91-101 (1947)

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VII. Model Experiments in Peptide Bond Synthesis

Since the problem of peptide bond synthesis is complicated in the case of proteins, which are large molecules of unknown structure, many attempts have been made to study peptide bond synthesis in simpler molecules, i e , in so-called "model" systems There have been two types of systems studied. (1) those in which actual net synthesis of peptide bonds occurred, and (2) those in which there were exchange or transfer reactions, without a net increase of peptide bonds in the system The former type will be considered first

Bergmann has carried out a brilliant series of experiments in which various derivatives of amino acids are condensed enzymatically to form peptide bonds For example, carbobenzoxyglycine anilide was formed in 80% yield from carbobenzoxyglycine and aniline by incubation at 40° and pH 4 6 with activated papain Similarly acetyl, benzoyl, and carbobenzoxy derivatives of alanine, leucine, and phenylalanine gave with aniline and phenylhydrazine the corresponding anilides and phenylhydrazides, acetylphenylalanylglutamic acid and *p*-toluenesulfonylglycine gave with aniline the corresponding anilides However, although hippuric acid condensed with aniline to give hippuric acid anilide, there was no synthesis of the amide from hippuric acid and ammonia [121, 122]

[118] P P Cohen and R W McGilvery, *J Biol Chem* **171**, 121–133 (1947)
[119] H Borsook and J W Dubnoff, unpublished observations
[120] H Chantrenne, *J Biol Chem* **189**, 227–233 (1951)
[121] M Bergmann and H Fraenkel-Conrat, *J Biol Chem* **119**, 707–720 (1937)
[122] M Bergmann and H Fraenkel-Conrat, *J Biol Chem* **124**, 1–6 (1938)

This latter observation was repeated by Waldschmidt-Leitz and Kuhn,[123] who found that condensation with hippuric acid occurred with aniline, *o*-, *m*-, and *p*-toluidine, *o*- and *p*-aminophenol, *o*-anisidin, *p*-aminobenzoic acid, sulfanilamide, and *o*- and *p*-phenylenediamine, but not with N-methylaniline, *o*-aminobenzoic acid, sulfanilic acid, α-aminopyridine, adenine, benzylamine, cyclohexamine, and ammonia

An explanation of this apparent anomaly comes from calculations of the free energies of formation For example, the value of the free energy of formation of hippurylanilide is approximately 5000 cal. at 37°, whereas the free energy of formation of amides and of small peptides is of the order of magnitude of −3500 cal. In other words, the formation of the anilide proceeds spontaneously, but the formation of peptides and amides does not Such vast differences in free energy changes make it impossible to transfer indiscriminately results from model experiments directly to the problem of protein biosynthesis.

A series of similar condensations involving amino acid derivatives was also carried out by Bergmann [122, 124, 125, 126] These include reactions, such as the condensation by papain of benzoylleucine and leucine anilide to form benzoylleucylleucine anilide, of benzoylphenylalanine and leucine anilide to form benzoylphenylalanylleucine anilide, of carbobenzoxyphenylalanylglycine and tyrosine amide to form carbobenzoxyphenylalanylglycyltyrosine amide, and by chymotrypsin of benzoyltyrosine and glycine anilide to form benzoyltyrosylglycine anilide and of benzoyltyrosine and leucine anilide to form benzoyltyrosylleucine anilide.

To some extent, the formation of the above peptides in measurable amounts is promoted by their relative insolubility. Similar but more soluble peptides have been postulated as quasi-intermediates to explain some experimental data For example, glycylleucine is not hydrolyzed by papain except in the presence of acetylphenylalanylglycine,[124] acetylphenylalanylglycylglycylglycylleucine is postulated as a soluble intermediate which hydrolyzes to give acetylphenylalanylglycine, glycine, and leucine In support of this is the fact that carbobenzoxyphenylalanylglycine and glycine anilide give the insoluble carbobenzoxyphenylalanylglycylglycyl anilide, which is insoluble and is not further hydrolyzed However, the differences in the behavior of the intermediates in these two cases may be due not only to differences in their solubilities or to differences in enzyme specificity but also to differences in the free energy changes involved in the subsequent reactions

A "peptide" synthesis which can be measured readily, *in vitro* or *in vivo*,

[123] E Waldschmidt-Leitz and K Kuhn, *Z physiol Chem* **285**, 22–35 (1950)
[124] M Bergmann and J S Fruton, *Ann N Y Acad Sci* **45**, 409–443 (1944)
[125] M Bergmann and J S Fruton, *J Biol Chem* **124**, 321–329 (1938)
[126] M. Bergmann and O K Behrens, *J Biol Chem* **124**, 7–10 (1938)

and which requires energy for the synthesis, is the condensation of benzoic acid with glycine to form hippuric acid. Hippuric acid has long been known to be formed in animals from benzoic acid and glycine; it was first synthesized *in vitro* in 60 to 75% yield, using kidney or liver slices as the enzyme source.[83] Since the thermodynamic equilibrium point is at less than 1% synthesis, such a high yield of hippuric acid could have been obtained only by coupling with an energy-yielding reaction. Indirect evidence was obtained that ATP was involved, but, as previously stated (see p. 205) phosphorylated intermediates did not seem to have been formed. Sarkar *et al.*[127] found that similar reactions are catalyzed by an enzyme in rat liver mitochondria, in which benzoic acid is replaced by some other aromatic or heterocyclic carboxylic acids and glycine by alanine or taurine. Cohen and McGilvery[118, 128, 129] studied the synthesis of *p*-aminohippuric acid from *p*-aminobenzoic acid and glycine, and showed that ATP, in the absence of oxidizable metabolite, promoted the synthesis anaerobically. They also studied the synthesis of ornithuric acids from α- and δ-benzoylornithine and *p*-aminobenzoic acid, using an acetone powder of the sedimentable macro-particles of chicken liver homogenate as enzyme, and found that the conditions for the synthesis were the same as in the *p*-aminohippuric acid synthesis.[130] CoA is necessary for the synthesis of hippuric acid;[120] the rôle of ATP probably, is in the formation of benzoyl-CoA which reacts with glycine to form hippuric acid and CoA.

Johnston and Bloch[131] studied the synthesis of a naturally occurring peptide, glutathione, using labeled glycine and glutamic acid as tracers. The pigeon liver enzyme preparation used contained, in a crude state, both a glutathione-synthesizing and a glutathione-hydrolyzing enzyme; on purification, an active preparation, from which the hydrolyzing enzyme was absent, could be obtained. Evidence was presented, that, under certain conditions, a net synthesis of glutathione occurred, i.e., the extent of incorporation of labeled amino acids was several times greater than could be accounted for by amino acid exchange in the glutathione originally present in the enzyme preparation. The enzyme system requires phosphate, magnesium, and ATP.

Speck[133, 134, 135] and Elliott[136, 137] simultaneously and independently stud-

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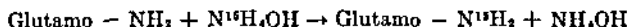
Chem 285, 22–35 (1950)

45, 409–443 (1944)

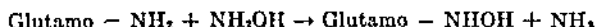
321–329 (1938)

[126] M Bergmann and O K Behrens, *J Biol Chem* 124, 7–10 (1938)

catalyzed by an enzyme in the cell extracts of a number of microorganisms:



$$\text{Glutamo} - NH_2 + N^{15}H_2OH \rightarrow \text{Glutamo} - N^{15}H_2 + NH_4OH$$

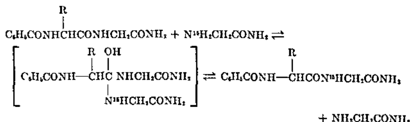


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The reaction is limited to asparagine and glutamine It was not inhibited by cyanide, fluoride or iodoacetate ATP and ADP had no effect, but this independence may be caused by insufficient purification of the enzyme system Extracts of mammalian tissues were also active

Stumpf *et al*[144, 145, 146] found a similar enzyme in pumpkin seedlings. After sufficient purification, the enzyme required ATP or ADP, manganous ion, and arsenate or phosphate With this enzyme only glutamine and not asparagine was active The evidence reported by Waelsch *et al* and of Stumpf *et al* indicates that the energy in pyrophosphate bonds of ATP and ADP are not used, that these compounds are required for some other reason

Fruton has proposed that the mechanism of the transfer reactions involves "activation" of the carbonyl bond in such a way that there is addition of ammonia, or of hydroxylamine, or of water When there is addition of water, subsequent splitting brings about hydrolysis, with addition of ammonia or of hydroxylamine, the result is replacement Strong evidence in favor of this mechanism comes from a study of what has been termed a transpeptidation reaction[147] Benzoyltyrosylglycine amide was incubated, in the presence of chymotrypsin, with glycine amide containing N^{15} in the amino group After 42% of the benzoyltyrosylglycine amide had been hydrolyzed, it was found that 17% of the nitrogen in the tyrosylglycine peptide bond of the remaining unhydrolyzed benzoyltyrosylglycine amide was replaced by N^{15} This reaction can be formulated as follows, the postulated intermediate being enclosed in brackets



$$C_6H_5CONHCH(R)CONHCH_2CONH_2 + N^{15}H_2CH_2CONH_2 \rightleftharpoons \left[C_6H_5CONH{-}CH(R)C(OH)(N^{15}HCH_2CONH_2)NHCH_2CONH_2 \right] \rightleftharpoons C_6H_5CONH{-}CH(R)CON^{15}HCH_2CONH_2 + NH_2CH_2CONH_2$$

[144] P K Stumpf and W D Loomis, *Arch Biochem* **25**, 451–453 (1950)

[145] P K Stumpf, W D Loomis, and C Michelson, *Arch Biochem* **30**, 126–137 (1951)

[146] C C Delwiche, W D Loomis, and K Stumpf, *Arch Biochem* **33**, 333–338 (1951)

[147] R B Johnston, M J Mycek, and J S. Fruton, *J Biol Chem* **187**, 205–211 (1950)

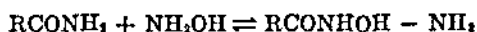
ied the synthesis of glutamine from glutamic acid and ammonia. The free energy of formation of the amide bond is of the same order of magnitude as that of simple peptides The reaction depends on ATP; inorganic phosphate is liberated in an amount equivalent to the amide synthesis. Only the γ-carboxyl group of glutamic acid undergoes the reaction.

The second type of model experiments include reactions in which transfer occurs, i e, a new peptide bond is formed, but there is no net increase in the total number of peptide bonds in the system.

The first example of this type of reaction was discovered by Bergmann: when hippurylamide is hydrolyzed by papain in the presence of aniline, hippuric acid anilide is formed faster than it would be formed from the hippuric acid and aniline present.[121] This fact indicates some direct replacement of the amide group by the anilide group. Another example is the formation of benzoylleucine anilide from benzoylleucine and glycine anilide, involving the direct replacement of the glycine residue by benzoylleucine.[122]

Fruton and his collaborators have reinvestigated this type of transfer reaction and have extended it. They found that, in the digestion of benzoylglycineamide by papain in the presence of $N^{15}H_3$, there was partial replacement of the amide group of the still unhydrolyzed benzoylglycineamide by $N^{15}H_3$ The degree of replacement was, however, always much less than the degree of hydrolysis; the highest degree of replacement reported was 3 5%, after 41% hydrolysis had occurred[138, 139] When there was no hydrolysis, there was no replacement The optimum pH for replacement was several pH units higher than for hydrolysis

A reaction that is somewhat analogous to these transamidation reactions is the papain-catalyzed exchange of the amide group of acylamino acid amides with hydroxylamine, forming hydroxamic acids:



$$RCONH_2 + NH_2OH \rightleftharpoons RCONHOH - NH_3$$

Here again, the hydroxamic acid formed was always a small fraction of the ammonia liberated by hydrolysis from the amide Cathepsin could also be used as enzyme.

Similar types of transamidation reactions were studied by Waelsch and later by Stumpf. Waelsch *et al*[140-143] found that the following reactions were

[138] J S. Fruton, *Yale J Biol and Med.* **22**, 263–271 (1950)

[139] R B Johnston, M J. Mycek, and J S Fruton, *J. Biol. Chem* **185**, 629–641 (1950)

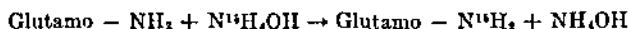
[140] H Waelsch, Borek, E , N. Grossowicz, and M. Schou, *Federation Proc.* **9**, 242 (1950)

[141] H. Waelsch, P Owades, E Borek, N Grossowicz, and M Schou, *Arch Biochem* **27**, 237–239 (1950).

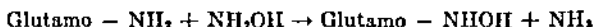
[142] H Waelsch, *Federation Proc* **10**, 266 (1951).

[143] H Waelsch, *Advances in Protein Chem.* **6**, 299–341 (1951)

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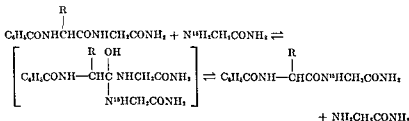


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Still another type of transfer reaction occurring during hydrolysis was reported by Hanes *et al.*[148] The enzyme, found in acetone powder of pig kidney or in fresh ox pancreas, acts on a mixture of glutathione with leucine, phenylalanine, or valine; during the hydrolysis of the glutathione, small amounts of the γ-glutamylpeptides of the above amino acids are formed Although it is difficult to estimate from the published data, it would appear that the amount of glutathione hydrolyzed is greater than the amount of γ-glutamylpeptide formed.

Zamecnik and Frantz[48] and Frantz and Loftfield[149] have studied the hydrolysis of glycylglycine by either a dipeptidase or a carboxypeptidase in the presence of C^{14}-labeled glycine They found that, if the reaction is stopped short of equilibrium, the amount of labeled glycine in the unhydrolyzed glycylglycine is greater than the calculated equilibrium amount. As in the previous cases, the amount of replacement in the unhydrolyzed dipeptide was small.

Another type of enzymatic synthesis of peptides has been found by Brenner *et al.*[150, 151] They incubated aliphatic or aromatic esters of DL-methionine or DL-threonine with chymotrypsin (which is a protease as well as an esterase[152-156]) and followed the reaction by filter paper chromatography They found that during the hydrolysis of the ester a large amount of a mixture of peptides of varying molecular weight was formed For example, beginning with DL-methionine-isopropyl ester they obtained L-methionyl-L-methionine and also the corresponding tripeptide; higher peptides that were formed were not identified.[157] The reaction presumably stops when the products attain such high molecular weights that they become insoluble No peptide formation occurs with only free methionine in the reaction mixture In no case did the authors observe peptide synthesis *without the simultaneous appearance of free amino acids* In the case of methionine esters, one part by weight of peptide was formed for every two parts by weight of methionine liberated as the free amino acid With threonine esters relatively more peptide is formed, with phenylalanine, tyrosine,

148

149

150 91

(1950)

151 M Brenner, E Sailer, and K Rufenacht, *Helv Chim Acta* **34**, 2096–2102 (1951)

152 G W Schwert, H Neurath, S Kaufman, and J E Snoke, *J Biol Chem* **172**, 221–239 (1948)

153 S Kaufman, H Neurath, and G W Schwert, *J Biol Chem* **177**, 793–814 (1949)

154 S Kaufman and H Neurath, *Arch Biochem* **21**, 245–246 (1949)

155 J E Snoke and H Neurath, *Arch Biochem* **21**, 351–362 (1949)

156 S Kaufman and H Neurath, *Arch Biochem* **21**, 437–453 (1949)

157 M Brenner and R W Pfister, *Helv Chim Acta* **34**, 2085–2096 (1951)

and tryptophan esters, relatively more free amino acids are liberated The authors believe that in the course of hydrolysis of the ester the amino group of an amino acid ester replaces the alkyl group being split off Resumption of hydrolysis at the ester bond of the newly formed peptide ester results in lengthening the peptide chain

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Fatty acids, largely in the form of esters with glycerol, cholesterol, and other alcohols, or combined with phosphoric acid, nitrogenous bases or carbohydrate, are vital components of all living cells Lipid metabolism is concerned with the assimilation, synthesis, utilization, and replacement of these various lipid molecules in biological systems. Our knowledge of the chemistry and physics of many of these lipids is still incomplete, but a considerable amount of detailed information will be found in the treatises on this subject by Hilditch[1] and Deuel [2] Since an appreciation of the peculiar properties of lipids is essential to the understanding of their metabolism, some of the more important chemical and physical characteristics must be briefly outlined.

I. Chemical and Physical Considerations

1 Some Important General Properties of Lipids

a. Solubility. The majority of lipids are practically insoluble in water. Short-chain fatty acids up to butyric, however, are freely soluble, and caproic acid will form a 1% solution in water at 30° The longer-chain fatty acids are progressively less soluble Many phospholipids such as lecithins, sphingomyelins, and lipositols form colloidal solutions or emulsions in water. Lipids are freely soluble in many organic solvents such as ethyl ether, acetone, chloroform, and benzene There are, however, marked differences in the solubility of individual members of the group, and great care must be exercised in the choice of solvents for extraction. The efficacy of solvent extraction may be profoundly altered by temperature, substitution in the fatty acid chain, salt formation, the presence of other lipids, or complex formation

Because of their solvent characteristics, special staining techniques can be used for the visualization of lipids in cells and tissues. Most of the histo-

[1] T P Hilditch, The Chemical Constitution of Natural Fats, Chapman & Hall, London, 1947

[2] H J. Deuel, Jr , The Lipids, Interscience Publishers, New York, 1951

CHAPTER 7

Lipid Metabolism

A. C FRAZER

Department of Pharmacology, The University of Birmingham, England

Fatty acids, largely in the form of esters with glycerol, cholesterol, and other alcohols, or combined with phosphoric acid, nitrogenous bases or carbohydrate, are vital components of all living cells Lipid metabolism is concerned with the assimilation, synthesis, utilization, and replacement of these various lipid molecules in biological systems Our knowledge of the chemistry and physics of many of these lipids is still incomplete, but a considerable amount of detailed information will be found in the treatises on this subject by Hilditch[1] and Deuel [2] Since an appreciation of the peculiar properties of lipids is essential to the understanding of their metabolism, some of the more important chemical and physical characteristics must be briefly outlined

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chemical methods which are alleged to differentiate between the various lipids in tissue preparations are not reliable, and more useful information may be gained by combining microanalysis and histochemistry.[3-7]

b. Surface Activity. Although many lipids are water-insoluble, they may have hydrophilic groups in the molecule which cause them to be preferentially orientated at an air-water or oil-water interface This orientation may have a number of effects—it may alter the chemical reactivity of the lipid molecule concerned, and it may play an important part in complex formation. The formation of a film of orientated lipid molecules at the interface may result in marked changes in interfacial tension and surface properties, with consequent alteration of the physical state of dispersion of the aqueous and non-aqueous phases in each other. The study of molecular interactions at the oil-water interface constitutes an important part of the background to lipid metabolism[8, 9]

c. Complex Formation. Although true ester formation occurs between fatty acids and many other substances, in some cases the molecular association appears to be of a more physical than chemical nature. Thus, complexes may be formed between lipids and bile salts, urea, starch, proteins, and other substances. The complexes formed between lipids and proteins have received considerable attention These complexes are often quite unstable and difficult to handle, and for this reason reliable evidence on their occurrence and structure can be built up only by repeated checking and crosschecking of data, using different methods of study Several of these complexes have now been isolated from various tissues—those showing essentially protein characteristics have been called **lipoproteins,** and those with lipid properties **proteolipids.** There seems little doubt that these molecular complexes, in which lipids are so frequently involved, play an important part in tissue ultrastructure.[10, 11]

d. Isomerism. The chemical and physical properties of fatty acid isomers *may vary greatly It is hardly surprising, therefore, that positional isomerism* in fatty acids with double bonds or substituted groups, or ***cis-trans*** isomerism in unsaturated fatty acids, may modify their nutritional value

[3] L Lison, Histochemie animale, Gauthier-Villars, Paris, 1936

[4] C Jackson, *Onderstepoort J Vet Sci Animal Ind* **19**, 169 (1944)

[5] J R Baker, Cytological Techniques, Methuen, London, 1950

[6] D Glick, Techniques of Histo- and Cyto-Chemistry, Interscience Publishers, New York, 1949

[7] K. Schmidt-Nielsen, *Compt rend trav lab. Carlsberg Sér chim* **24**, 233 (1942); **25**, 97 (1944)

[8] N. K. Adam, The Chemistry and Physics of Surfaces Oxford University Press, London, 1941

[9] J. H. Schulman, P Doty, and R Matalon, *Discussions Faraday Soc* **6**, 21 (1949)

[10] J A Lovern, *Dept Sci Ind Research (Brit), Food Invest. Special Rept* 52, 1942.

[11] E. Chargaff, *Advances in Protein Chem* 1 1 (1944)

e. Crystallization. Many lipids have a crystalline structure which can be studied and characterized by X-ray diffraction [12] An ordered structure may be found in the lipids of some living tissues so that X-ray diffraction can also be used in suitable cases to elucidate tissue ultrastructure [13] The polarizing microscope is also of value in this type of investigation.

f. Melting Point. Saturated fatty acids up to and including undecanoic acid are liquid at human body temperature (37°), but those with 12 or more carbons are solid Odd-chain fatty acids have a lower melting point than the even-chain acid with 1 carbon less The presence of double bonds results in a marked lowering of the melting point With *cis* forms the melting point progressively decreases as the double bond moves away from the carboxyl group, but with *trans* forms this effect is not seen In polyethenoid acids the melting point is higher in conjugated than in non-conjugated acids The *trans* form of an unsaturated acid has a higher melting point than the *cis* form, provided the structure is otherwise similar Methyl substitution in fatty acids causes a lowering of melting point Fatty acids may form eutectic mixtures that tend to crystallize in certain fixed proportions with a characteristic melting point For fuller details of the properties of fatty acids, reference may be made to the treatise by Markley [14]

g. Spectral Properties. Infrared and ultraviolet absorption spectroscopy and the study of the Raman spectra have proved of value in the study of the structure of lipids—especially unsaturated fatty acids [15, 18]

h. Oxidative Rancidity. Many fats, especially of the more unsaturated series, undergo oxidative rancidity in the presence of air This action may be catalyzed by heat, light, various metals, or the presence of traces of other rancid fats In the course of the oxidation, peroxides and a wide range of other derivatives are formed Polymerization may occur, with a great increase in viscosity [19, 20]

2 Main Groups of Lipids of Biochemical Interest

a. Fatty Acids. With a few exceptions, natural fatty acids are straight-chain aliphatic compounds with an even number of carbons They may be conveniently considered from the biochemical point of view in five groups

[12] A Muller, *Transactions Faraday Soc* **25**, 347 (1929)
[13] F O Schmitt, R S Bear, and G L Clark, *Radiology* **25**, 131 (1935)
[14] K S Markley, Fatty Acids, Interscience Publishers, New York, 1947
[15] R B Barnes, R C Gore, U Liddel, and V Z Williams, Infrared Spectroscopy, Reinhold Publishing Corp, New York, 1944
[16] J P Kass, *Protective and Decorative Coatings* **4**, 362 (1944)
[17] R H Barnes, I I Rusoff, E S Miller, and G O Burr, *Ind Eng Chem Anal Ed* **16**, 385 (1944)
[18] J W McCutcheon, M F Crawford, and H L Welch, *Oil & Soap* **18**, 9 (1941)
[19] C H Lea, *Dept Sci Ind Research (Brit.) Food Investigation Rept* 46, 1938
[20] W O Lundberg, *Hormel Institute Publication* 20 (1947)

(1) *Saturated Volatile Fatty Acids.* This series starts with acetic and ends with capric acid—the lowest members are freely water-soluble, and their solubility decreases with increasing chain length. All are liquid at mammalian body temperatures and can be separated from other fatty acids by steam distillation Acetic, propionic, and butyric acid have little surface activity and most commonly arise from the breakdown of carbohydrates or longer chain fats The other members of the series occur in milk fats and some vegetable oils.

(2) *Saturated Non-Volatile Fatty Acids.* This series starts at lauric acid and includes some long-chain fatty acids with 30 or more carbons. These fatty acids are all solid at mammalian body temperatures. They are practically insoluble in water but soluble in many organic solvents. They have considerable surface activity, and their molecules may be preferentially orientated at an oil-water interface. The commonest natural acids in this group are myristic, palmitic, and stearic, which occur in many fats The longer chain acids (20 to 30 carbons) are found in small quantities in fats and form a major component of many natural waxes

(3) *Unsaturated Monoethenoid Fatty Acids.* The most important of this group is oleic acid (cis-Δ-9-octadecenoic acid) which is the commonest of all natural fatty acids, often forming more than 50% of the total fatty acids and rarely less than 10%. The melting point of this acid is below mammalian body temperature It is preferentially orientated at the oil-water interface and forms complexes with bile salts and starch. It is found in almost all natural fats, especially in warm-blooded animals, its place is taken to some extent in cold-blooded animals by palmitoleic acid (Δ-9-hexadecenoic acid)

(4) *Unsaturated Polyethenoid Fatty Acids.* The properties of these unsaturated acids vary considerably according to the number and relative positions of the double bonds.

Linoleic acid (Δ-9,12-octadecadienoic acid) is the commonest of the polyethenoid acids and is found principally in vegetable oils. It occurs in much smaller quantities in many animal fats, but none is found in butter or marine fats Linolenic acid (Δ-9,12,15-octadecatrienoic acid) is also found largely in vegetable oils Both these fatty acids are dietary essentials Arachidonic acid (Δ-5,8,11,14-eicosatetraenoic acid) is found exclusively in animal fats, especially phospholipids The natural acids, linoleic, linolenic, and arachidonic, are fluid at body temperature Conjugated position of the double bonds gives a compound with a much higher melting point Eleostearic acid (Δ-9,11,13-octadecatrienoic acid) is a conjugated acid present in vegetable oils, and it has a melting point above mammalian body temperature The unsaturated fatty acids form complexes with urea and proteins. They are readily oxidized in the presence of air.

(5) *Substituted Fatty Acids.* A number of hydroxy acids are known to

occur in nature, the best known being ricinoleic acid in castor oil A number of others also occur, especially in wool fats Methyl-substituted fats also occur naturally—isovaleric acid in certain fish fats, and a number of branch-chained fats in tubercle and other bacilli and in wool wax Halogenated fats have been used as tracers by many workers, but it should be remembered that the properties of such compounds may differ considerably from the original fat Fatty acids labeled with deuterium or with C^{14} are now being employed extensively in studies on lipid metabolism The range of C^{14} labeled fatty acids has so far been restricted to the saturated series

b. Glycerides. Fatty acids occur most commonly in nature as esters of glycerol The commonest form is the triglyceride, but diglycerides and monoglycerides also occur, especially in the lumen of the intestine. The

TABLE 1

MOLECULAR COMPONENTS OF MAIN PHOSPHOLIPIDS AND CEREBROSIDES

			Lipid			Base			Carbohydrate	
	Phosphoric acid	Glycerol	Fatty acids	Polyethenoid fatty acids	Fatty aldehydes	Choline	Ethanolamine or serine	Sphingosine	Inositol	Galactose or glucose
Lecithins	+	+	+	+	−	+	−	−	−	−
Cephalins	+	+	+	(+)	−	−	+	−	−	−
Lipositols	+	+	+	−	−	−	(+)	−	+	−
Plasmalogens	+	+	−	−	+	−	+	−	−	−
Sphingomyelins	+	−	+	−	−	+	−	+	−	−
Cerebrosides	−	−	+	−	−	−	−	+	−	+

majority of natural glycerides contain mixed fatty acids, lauric, myristic, palmitic, stearic, and oleic being the commonest in land animals, whereas more unsaturated fatty acids occur in fish glycerides The glycerides are soluble in the usual range of organic solvents, but insoluble in water Polymerization of glycerides containing unsaturated fatty acids occurs under certain conditions of heating and oxidation, which may affect the nutritional value and possible toxicity of the oils The lower glycerides have marked surface active properties and are excellent emulsifying agents The greatest quantity of lipid in most animals and plants occurs as glycerides.

c. Phospholipids. Phosphorus-containing lipids are of the greatest biological importance, and our knowledge of their structure and functions is still rudimentary The composition of some of the more important phospholipids is indicated in Table 1

(1) *Monaminophosphatides* These are [illegible]

diglyceride anc[illegible]

in the cephalir[illegible]

portion vary widely—a saturated lecithin has been isolated from many tissues which contains only palmitic acid, but many other lecithins contain highly unsaturated fatty acids, especially arachidonic acid.

Lecithin is soluble in most organic solvents, but insoluble in acetone. It forms colloidal solutions in water. It forms complexes with bile salts, inorganic salts, sugars, and proteins. Cephalins differ from lecithins also in their solubility in different solvents. They also oxidize on exposure to air and form complexes with proteins. Lecithins and cephalins occur in all cells, there are less cephalins in blood plasma than lecithins.

(2) *Inositol Phosphatides.* The components of lipositol include inositol, phosphoric acid, saturated fatty acids, ethanolamine, and tartaric acid It is found in brain tissue and also in soybean phosphatides

(3) *Plasmalogens.* These lipid components of the ground substance of cytoplasm are acetals of fatty aldehydes combined with ethanolamine glycerophosphate They are insoluble in water, but freely soluble in chloroform Owing to their aldehyde content, their presence in tissues can be detected by the Schiff reaction.[21]

(4) *Diaminophosphatides. Sphingomyelins.* These are non-glycerol-containing phospholipids; acid amides of sphingosine with fatty acids in ester linkage with phosphorylcholine. The fatty acids are mainly long-chain saturated fatty acids, the commonest one being lignoceric acid—the two bases are choline and sphingosine It is possible that the sphingomyelin may also exist as an ester with palmitic or other fatty acids. The sphingomyelins emulsify in water but are insoluble in ether and acetone. Sphingomyelins are found in nervous tissue and also in kidney, liver, spleen, and the blood

d. Cerebrosides. Cerebrosides are closely related to the sphingomyelins, but contain no phosphorus. They consist of lignoceryl sphingosine linked to galactose or glucose, instead of to phosphorylcholine. They are insoluble in water, ethyl ether, and petroleum ether, but soluble in hot alcohol and pyridine Cerebrosides are found in the nervous tissue of mammals and birds and are probably present in many other tissues.

e. Cholesterol and Other Natural Alcohols. Cholesterol occurs in all cells, [illegible]

cursor of many steroid hormones, but from the point of view of lipid metabolism its structural and general metabolic properties will be mainly considered There is a high proportion of cholesterol in the skin and in sebum; in the surface of the skin it may be changed to vitamin D_3 by ultraviolet light. It also occurs in wool fats. It is water-insoluble, but soluble in most organic solvents

[21] R. Fuelgen, K Imhauser, and M. Behrens, *Z physiol Chem* 180, 161 (1929)

Long-chain aliphatic alcohols, usually containing an even number of carbons, occur naturally, especially in whale oil and insect and plant waxes Palmityl, stearyl, and oleyl alcohol may also be combined with glycerol to form glyceryl ethers which have been found in fish liver oils Many of the natural waxes contain fatty acids with more than 20 carbon atoms esterified with cholesterol or other alcohols

3 Anabolic and Catabolic Reactions Involving Lipids

The lipids undergo various changes in the tissues in the course of metabolism, and many of these changes were deduced some years ago from observations in animals and man under normal and abnormal conditions In recent times the availability of isotope-labeled molecules has made it possible to study these complex chemical changes in greater detail and to obtain more conclusive proof of the chemical pathways used in these anabolic and catabolic reactions Another field of advance has been the development of methods using isolated tissue preparations It should be remembered, however, that many of these experimental studies merely show what could happen in the body and not what does

a. Fatty Acids. (1) *Biosynthesis* Lawes and Gilbert[22] demonstrated in *1860 that fat was synthesized from carbohydrate in the pig In 1878* Nencki[23] suggested that this synthesis involved acetaldehyde The mechanism of synthesis from 2-carbon fragments was elaborated by Magnus-Levy (1901)[24] and Leathes (1906)[25] and Smedley and Lubrzynska (1913)[26] suggested that pyruvic acid might be involved in fat synthesis The use of isotope-labeled materials has shown that long-chain fatty acids can be built up by the condensation of 2-carbon units,[27] or by the addition of a 2-carbon unit to a longer fatty acid chain,[28] acetaldehyde being converted into fatty acids more efficiently than acetate [29] Fatty acids can be formed from a number of different starting materials, but most of them are probably first degraded to 2-carbon compounds [30] Pyruvic acid plays an important part in fat synthesis[31] and is the important intermediate in the interconversion of carbohydrate to fatty acid The intervention of carbohydrate is necessary to give an over-all increase of fatty acid, since the other main source of 2-carbon units is from the catabolism of fatty acid

[22] J B Lawes and J H Gilbert, *J Roy Agr Soc Engl* **21**, 433 (1860)
[23] M Nencki, *J prakt Chem* **17**, 105 (1878)
[24] A Magnus-Levy, *Berlin physiol Ges* **5** (1901)
[25] J B Leathes, Problems in Animal Metabolism, Murray, London, 1906
[26] E Lubrzynska and I Smedley, *Biochem J* **7**, 375 (1913)
[27] D Rittenberg and K Bloch, *J Biol Chem.* **154**, 311 (1944)
[28] I Zabin, *J Biol Chem* **189**, 355 (1951)
[29] R O Brady and S Gurin, *J Biol Chem* **189**, 371 (1951)
[30] R O Brady and S Gurin, *J Biol Chem* **186**, 461 (1950)
[31] K Bloch, *Cold Spring Harbor Symposia Quant Biol.* **13**, 29 (1948)

itself Pyruvate probably forms a 2-carbon compound by decarboxylation[32] before it takes part in fatty acid synthesis, and thiamine is a coenzyme in this decarboxylase system.[33]

These various reactions satisfactorily explain the formation of the common long-chain saturated fatty acids. There is some doubt, however, whether oleic acid, which is also synthesized, is necessarily derived from stearic acid by desaturation, or that it constitutes an intermediate in the formation of stearic acid[34] The isotope distribution in oleic acid, after administration of heavy water, is only 40% of that in the saturated acids,[35] but the distribution of isotopes in the two halves of the oleic acid chain indicate that it can be built up by condensation of 2-carbon units.[36] Studies in fat synthesis in bone marrow indicate the independent synthesis of saturated and unsaturated fatty acids[37] As Hilditch[1] has pointed out, the formation of polyene compounds from the condensation of acetaldehyde and crotonaldehyde[38] and their subsequent hydrogenation may have some relevance to oleic acid synthesis. In *L. casei* biotin appears to be related to oleic acid formation[39] The polyethenoid fatty acids do not appear to be synthesized in the tissues of land animals, and administered deuterium is not incorporated into them as it is into other fatty acids[40] Whether marine animals can obtain the large quantities of poly-unsaturated fatty acids found in their body lipids from dietary sources remains an open question

Although most recent work has provided major support for fatty acid synthesis from 2-carbon compounds, the possibility cannot be entirely excluded that other units, such as 3-carbon and 6-carbon compounds derived from carbohydrate,[41] may sometimes be involved This has been suggested as a possible explanation of the groupings in the unsaturated fatty acids,[1] and also for the formation of the long-chain saturated acids with 24 and 30 carbons However, the unsaturated acids, with the exception of oleic acid and a few other monoethenoid acids, are not synthesized in the animal body and, furthermore, carbon dioxide is always formed when hexoses are converted into fats,[42] which is clearly indicative of decarboxylation with formation of 2-carbon units Fatty acid synthesis in the liver is

32 R I Crandall and S Gurin, *J Biol Chem* **181**, 829 (1949)
33 E W McHenry and G Gavin, *J Biol Chem* **125**, 653 (1938), **128**, 45 (1939); **134**, 693 (1940)
34 [illegible] 1926
35 [illegible]
36 [illegible]
37 [illegible] -ta **7**, 460 (1951)
38 R Kuhn, C Grundmann, and H Trischmann, *Z physiol. Chem* **248**, 4 (1937)
39 W Trager, *J. Bact* **56**, 195 (1948)
40 K. Bernhard and R Schoenheimer, *J. Biol. Chem* **133**, 707 (1940)
41 E. F. Armstrong and J Allan, *J Soc Chem. Ind (London)* **43**, 216T (1924)
42 H Haehn and W. Kintoff, *Chem. d Zelle u Gewebe* **12**, 115 (1925)

markedly stimulated by insulin[30, 43] and is defective in diabetic liver[44, 45] but not in liver slices from a Houssay cat [46] The stimulating effect of insulin on lipogenesis has also been observed in adipose tissue[47] and in the mammary gland [48] Acetoacetate is not broken down in the liver and does not take part in fatty acid synthesis [49]

(2) *Hydrogenation* It has been shown that hydrogenation occurs in the rumen, and this accounts for the lack of linolenic acid in the depot fats of ruminants [50] Hilditch[51] has postulated a biohydrogenation in the "stearic-rich" animal fat depots of pigs, oxen, sheep, and goats Hydrogenation has been thought to account for the formation of the alcohol ethers in sharks and other fish [52]

(3) *Desaturation* For many years desaturation of fatty acids in the liver was considered to be the first vital step in fatty acid catabolism [53] The main evidence for this hypothesis was that fat tended to accumulate in the liver and that the liver lipids were more unsaturated than depot or dietary fats In recent years evidence has accumulated to show that desaturation in the liver is not a necessary step in fatty acid catabolism The difference in the degree of saturation of liver and depot fats can be readily accounted for on a basis of relative turnover rate of different fatty acids in the two tissues and the difference from the diet by processes of selection The unsaturated fatty acids in the liver are largely arachidonic acid,[54] which is metabolized extremely slowly, and linolenic and linoleic acids, which can hardly be intermediates in normal fat catabolism, since their replacement in the body is found to depend upon their inclusion in the diet [55] In fish the liver fats may be more saturated than those in the depots, and in all animals so far investigated saturated fatty acids are readily broken down in extrahepatic tissues, so that preliminary desaturation in the liver plays no essential part in their metabolism Finally, there appears to be no significant difference in the rate of oxidation of saturated and unsaturated fatty acids [56] For these reasons the view that preliminary desaturation of

[43] K Bloch and W Kramer, *J Biol Chem* **173**, 811 (1948)
[44] R O Brady and S Gurin, *J Biol Chem* **187**, 589 (1950)
[45] S S Chernick and I L Chaikoff, *J Biol Chem* **186**, 527, 535 (1950)
[46] R O Brady, F D W Lukens, and S Gurin, *Science* **113**, 413 (1951)
[47] A E Renold, A Marble, and D W Fawcett, *Endocrinology* **46**, 55 (1950)
[48] J H Balmain, T H French, and S J Folley, *Nature* **165**, 807 (1950)
[49] J M Buchanan, W Sakami, and S Gurin, *J Biol Chem* **169**, 411 (1947)
[50] R Reiser, *Federation Proc* **10**, 236 (1951)
[51] T. P Hilditch and W J Stainsby, *Biochem J* **29**, 90 (1935)
[52] J A Lovern, *Biochem J* **31**, 755 (1937)
[53] E L Kennaway and J B Leathes, *Lancet* **1**, 95 (1909)
[54] E Klenk and O Schonebeck, *Z physiol Chem* **209**, 112 (1932)
[55] O Burr and M M Burr, *J Biol. Chem* **82**, 345 (1929); *ibid* **86**, 587 (1930)
[56] E P Kennedy and A L Lehninger, *J Biol. Chem* **185**, 275 (1950).

fatty acids occurs in the liver, as a necessary step in fat oxidation, should be discarded

(4) *Degradation of Long-Chain Fatty Acids* Knoop (1906)[57] proposed that the mechanism of breakdown of long-chain fatty acids was by the progressive formation of 2-carbon fragments by β oxidation. Later, various alternative possibilities, such as multiple oxidation[58] and ω oxidation have[59] been proposed Recent work with isotope-labeled fatty acids strongly supports the view that fatty acids form 2-carbon,[60] and occasionally 4-carbon, units No differences were found between the oxidation of the first, sixth, and eleventh carbons of palmitic acid when these were labeled with C^{14}, so the whole fatty acid chain rapidly fragments [61] Degradation of long-chain fatty acids to 2-carbon units occurs in the liver and in many extrahepatic tissues, including heart, kidney, and skeletal muscle.[62, 63, 64] The total fatty acid oxidation in the extrahepatic tissues probably does not normally exceed 40% of the total.[65]

(5) *The 2-Carbon Units* Long-chain fatty acids give rise to at least two types of 2-carbon unit—one derived from the carboxyl end of the molecule and the other from the terminal 2 carbons. This terminal 2-carbon fragment tends to be mainly an acetylating unit.[66, 67] Consequently, the shorter-chain fatty acids are liable to form more acetoacetic acid than longer-chain acids if an adequate supply of oxaloacetate is present. The 2-carbon units formed from fatty acids may enter the tricarboxylic acid cycle by condensation with oxaloacetate to form citric acid,[68, 69] or it may be synthesized into fatty acid or cholesterol,[70] or finally two units may condense to form acetoacetic acid [71, 72] The removal of the 2-carbon compounds into the tricarboxylic acid cycle depends upon an adequate supply of oxaloacetate [73] Coupled

[57] F Knoop, *Beitr chem Physiol Path* **6**, 150 (1905)
[58] M Jowett and J H Quastel, *Biochem J* **29**, 2143, 2159, 2181 (1935).
[59] P E Verkade and J van der Lee, *Biochem J* **28**, 31 (1934)
[60] R Schoenheimer and D Rittenberg, *J Biol Chem* **120**, 155 (1937)
[61] E O Weinman, I L. Chaikoff, W G Dauben, M. Gee, and C Entenman, *J Biol Chem* **184**, 735 (1950)
[62] R P Geyer, M Cunningham, and J Pendergast, *J Biol Chem* **185**, 461 (1950)
[63] R P Geyer and M Cunningham, *J Biol Chem* **184**, 641 (1950)
[64] S Weinhouse, R H Millington, and M E Volk, *J. Biol. Chem* **185**, 191 (1950)
[65] D S Goldman, I L Chaikoff, W O Reinhardt, C Entenman, and W G Dauben, *J Biol Chem* **184**, 719 (1950)
[66] V. Lorber, M Cook, and J Meyer, *J. Biol. Chem* **181**, 475 (1949)
[67] D I Crandall, R O Brady, and S Gurin, *J Biol Chem* **181**, 845 (1949)
[68] F L Breusch, *Science* **97**, 490 (1943)
[69] H Wieland and C Rosenthal, *Ann* **554**, 241 (1943)
[70] [illegible]
[71] [illegible]
[72] ... 1940)
[73] [illegible]

oxidative phosphorylation is also thought to play a part in the metabolism of the 2-carbon fragment[74] and the site of the reaction in the liver cell appears to be the mitochondria [75]

(6) *Acetoacetate*. In Knoop's original conception of β oxidation of fats, the formation of acetoacetate was thought to arise from incomplete oxidation of the last 4 carbons of the fatty acid chain It is now known that this is not the case, but that acetoacetic acid is usually formed from condensation of the 2-carbon units, as originally suggested by Friedman [71, 72] From the distribution of isotopic carbon in the carbonyl and carboxyl carbons of acetoacetic acid, it has been shown that it can arise from both 2-carbon and 4-carbon units [76] Acetoacetic acid is not broken down in the liver,[77, 78] but it can be utilized by the tissues The extent to which acetoacetate and fatty acids are utilized depends upon their relative concentrations, since they both enter the tricarboxylic acid cycle in a similar manner If the passage of 2-carbon units into the tricarboxylic acid cycle in the liver is interfered with, acetoacetic acid formation may be greatly increased [79, 80] Since the liver is unable to break it down, the acetoacetate passes into the blood and, if the blood level exceeds 20 mg per 100 ml, ketonuria occurs [81] Much of the acetoacetic acid may be converted into β-hydroxybutyric acid and acetone Acetoacetate can also be utilized by the liver for cholesterol synthesis without previous degradation [29]

b. Cholesterol. (1) *Biosynthesis* It has been known for some years that there is a negative balance of cholesterol in most animals, the level of cholesterol in the body being maintained constant, even though excretion exceeds intake The extent of this negative balance in man has been calculated as 300 mg of cholesterol per day [82, 83, 84] That cholesterol synthesis occurs in the animal body is shown by the incorporation of deuterium into cholesterol after the injection of heavy water [70] Cholesterol cannot be synthesized, however, by certain insect larvae[85] and parasites,[86] and it is a dietary essential for these creatures It has now been conclusively demon-

[74] A L Lehninger and E P Kennedy, *J Biol Chem* **173**, 753 (1948)
[75] E P Kennedy and A L Lehninger, *J Biol Chem* **179**, 956 (1949)
[76] G Medes, S Weinhouse, and N F Floyd, *J Biol Chem* **157**, 35 (1945).
[77] I Snapper and A Grunbaum, *Biochem Z* **181**, 410 (1927)
[78] N Blixenkrone-Moeller, *Z physiol Chem* **252**, 117 (1939)
[79] R H Barnes, E M MacKay, G K Moe, and M B Visscher, *Am J Physiol* **123**, 272 (1938)
[80] A L Lehninger, *J Biol Chem* **143**, 147 (1942)
[81] H E Martin and A N Wick, *J Clin Invest* **22**, 235 (1943)
[82] H J Channon, *Biochem J* **19**, 424 (1925)
[83] [illegible]
[84] [illegible]
[85] [illegible]
[86] [illegible]

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57 F Knoop, *Beitr chem Physiol Path.* 6, 150 (1905)
58 M. Jowett and J H Quastel, *Biochem J.* 29, 2143, 2159, 2181 (1935)
59 P E. Verkade and J van der Lee, *Biochem J* 28, 31 (1934).
60 R Schoenheimer and D Rittenberg, *J Biol Chem* 120, 155 (1937)
61 E O Weinman, I L Chaikoff, W G Dauben, M Gee, and C. Entenman, *J Biol Chem* 184, 735 (1950)
62 R P Geyer, M Cunningham, and J Pendergast, *J Biol Chem* 185, 461 (1950)
63 R. P Geyer and M Cunningham, *J Biol Chem* 184, 641 (1950)
64 S Weinhouse, R. H Millington, and M E Volk, *J Biol Chem* 185, 191 (1950)
65 D S Goldman, I L Chaikoff, W O Reinhardt, C Entenman, and W G Dauben, *J Biol Chem.* 184, 719 (1950)
66 V Lorber, M Cook, and J Meyer, *J Biol. Chem* 181, 475 (1949)
67 D I Crandall, R O Brady, and S Gurin, *J. Biol Chem* 181, 845 (1949)
68 F. L Breusch, *Science* 97, 490 (1943)
69 [illegible]
70 [illegible]
71 [illegible]
72 [illegible] 3 (1940)
73 [illegible]

hormones, and possibly estrogens[98-103] Irradiation of the sterol molecule may cause the ring to break between the ninth and tenth carbons, and such substances may exhibit vitamin D activity.[104]

(3) *Esterification* Cholesterol in blood is partly esterified. The cholesterol in the nervous tissue is free, but esters appear during degeneration Cholesterol esterases have been described in intestinal juices, blood, and[105, 106, 107] tissues, but their significance is still obscure

(4) *Destruction and Excretion* Cholesterol can be destroyed in the body, and this destruction appears to be enhanced if the dietary cholesterol is increased[108] The half-life of cholesterol was found to be 6 days in the liver and 31 to 32 days in the carcass in rats,[109] 3 days in the liver and 1 5 days in the intestine in rabbits,[92] and 8 days has been estimated as the half-life of cholesterol in man[110] Cholesterol forms cholestenone in the body, and this is excreted in the bile This substance is partly reabsorbed, and the remainder is broken down to coprosterol by intestinal organisms.[98, 99, 100] In familial xanthomatosis the ability to eliminate cholesterol is impaired.[111, 112]

c. Glycerides. (1) *Biosynthesis* Fatty acids usually occur in animal tissues esterified with glycerol, or occasionally other alcohols The glycerol triesters can be synthesized in intestinal cells,[113] mammary gland,[114] and adipose tissue[115] Glycerol for the synthesis in mammary gland can apparently be derived from glucose

(2) *Hydrolysis* The triesters of glycerol with long-chain fatty acids are hydrolyzed by lipases, but they are relatively resistant to the action of esterases, which split short-chain fatty esters, and they are not acted upon by lecithinases or cholesterol esterases Pancreatic lipase is said to have the

[98] I H Page and W Menschick, *Naturwissenschaften* **18**, 585 (1930)
[99] O Rosenheim and T A Webster, *Biochem J* **37**, 513 (1943)
[100] R Schoenheimer, D Rittenberg, and M Graff, *J Biol Chem* **111**, 183 (1935).
[101] K Bloch, *J Biol Chem* **157**, 661 (1945)
[102] C N H Long, *Federation Proc* **6**, 461 (1947)
[103] L Claesson and N A Hillarp, *Acta Physiol Scand* **13**, 115 (1947)
[104] C E Bills, *J Am Med Assoc* **110**, 2150 (1938)
[105] S J Thannhauser, *Deut Arch klin Med* **141**, 290 (1923)
[106] W M Sperry, *J Biol Chem* **111**, 407 (1935)
[107] E le Breton and J Panteléon, *Arch sci physiol* **1**, 199 (1947)
[108] I H. Page and W Menschick, *J Biol Chem* **173**, 811 (1932)
[109] A Pihl, K Bloch, and H S Anker, *J Biol Chem* **183**, 441 (1950)
[illegible]
[illegible] rk, 1949
[illegible] ne, Longmans Green & Co , London, 1936
[114] T H French and G Popják, *Biochem J* **49**, iii (1951)
[115] B Shapiro and E Wertheimer, *J Biol Chem.* **173**, 725 (1948)

strated that cholesterol may be largely synthesized by mammals from 2-carbon fragments Acetate, acetaldehyde, acetone, isovalerate, acetoacetate, pyruvate, and short-chain fatty acids can all act as precursors for cholesterol synthesis Some of these precursors, such as acetoacetate and isovalerate, can form cholesterol without degradation to 2-carbon compounds Isovalerate breaks down to a 2-carbon and a 3-carbon fragment and the latter may form either a 2-carbon unit, similarly to acetone, or a 4-carbon compound by carbon dioxide fixation. Long-chain fatty acids do not act as direct precursors of cholesterol [29, 30, 87, 88, 89] From degradation studies of synthesized cholesterol molecules, it appears that any of the carbons in cholesterol can be derived from acetic acid [90, 91]

Cholesterol synthesis occurs in the liver and the extrahepatic tissues Nervous tissue does not appear to play an active part in cholesterol metabolism, but synthesis has been demonstrated in the intestines, ovaries, mammary glands, adrenals, and skin, and in the fetal rabbit. Homogenization causes a marked decrease in the cholesterol-synthesizing capacity of a tissue, which appears to depend upon the integrity of the cell [92-94] Cholesterol synthesis is not increased but depressed by the addition of glucose or pyruvate, it is not stimulated by insulin,[43] it is normal and may even be enhanced in the diabetic liver,[44] and it is stimulated by administration of cortisone,[96] but inhibited by the ingestion of a high cholesterol diet.[95]

(2) *Modification of Sterol Structure.* Small changes in the molecular structure of sterols may considerably alter their biological properties Thus, plant sterols are said not to be absorbed from the intestine,[97] although in view of the activity of the intestinal cells with regard to sterol metabolism this point requires further study Small differences in structure determine the widely differing emphasis in the biological effects of the various sterol hormones and the bile acids. It has been thought for some time that cholesterol is likely to be the mother substance from which the other sterols in the body are derived Some definite relationship of this sort has now been demonstrated with regard to bile salts, progesterone, adrenal cortical

[87] I Zabin and K Bloch, *J Biol Chem* **186**, 131 (1950), *ibid* **192**, 267 (1951)
[88] K Bloch and D Rittenberg, *J Biol Chem* **155**, 243 (1944)
[89] G L Curran, *J Biol Chem* **191**, 775 (1951)
[90] K Bloch and D Rittenberg, *J Biol. Chem* **145**, 625 (1942)
[91] H N Little and K Bloch, *J Biol Chem* **183**, 33 (1950)
[92] G Popják and M L Beeckman, *Biochem J* **46**, 547 (1949), *ibid* **47**, 237 (1950)
[93] K Bloch, D Rittenberg, and B N Berg, *J Biol Chem* **149**, 511 (1943)
[94] K Bloch, E Borek, and D Rittenberg, *J Biol Chem* **162**, 441 (1946)
[95] [illegible]
[96] [illegible] , 329 (1951)
[97] [illegible] 579 (1931)

enates. The cytoplasm of the cell appears to play more part than the nucleus.[131, 132]

In vitro studies have shown that phospholipid is synthesized by liver, kidney, small intestine, muscle, brain, and nerve No synthesis of phospholipid could be demonstrated in plasma [129, 133, 134] Phospholipid turnover can be measured by the use of P^{32}, but it is important to distinguish between the various types of phospholipids and to measure the turnover rate in the immediate precursor of the phospholipid studied [135] The synthesis of individual phospholipids appears to vary in different tissues and to be affected by the supply of raw materials In the brain more P^{32} passes into cephalins than into lecithins [136, 137] The administration of choline alters the proportions of lecithins and cephalins formed, and lack of unsaturated fatty acids, which are commonly found in lecithins, results in the use of more saturated acids, but in neither of these cases need the total phospholipid turnover be altered [138, 139] Thus the total phospholipid synthesis may continue unchanged, but the type of molecule produced and the biological effects it causes may be different. Some of the conclusions based on total phospholipid turnover should be carefully re-examined

(2) *Hydrolysis* Lecithin, glycerylphosphorylcholine and glycerophosphate differ greatly in their hydrolytic reactions Alkaline phosphatase does not split lecithin, but breaks down glycerylphosphorylcholine slowly and glycerophosphate extremely rapidly,[127] on the other hand, the latter substance is resistant to acid hydrolysis, whereas the others can be readily hydrolyzed by normal HCl Lecithin is not affected by lipases, but it can be broken down by lecithinases [140] Lecithinase A splits off one fatty acid, leaving lysolecithin, which has powerful hemolytic properties The enzyme occurs in a number of snake venoms [141, 142] Lecithinase B splits off both fatty acids, giving glycerylphosphorylcholine, and is found in wasp and bee

[131] G C Hevesy, *Nature* **156**, 534 (1945)
[132] G L Ada, *Biochem J* **45**, 422 (1944)
[133] M C Fishler, A Taurog, I Perlman, and I L Chaikoff, *J Biol Chem* **141**, 809 (1941)
[134] B A Fries, H Schachner, and I L Chaikoff, *J Biol Chem* **144**, 59 (1942)
[135] I L Chaikoff and D B Zilversmit, *Advances in Biol Med Physics* **1**, 321 (1948)
[136] E Chargaff, K B Olson, and P F Partington, *J Biol Chem* **134**, 505 (1940)
[137] G C Hevesy and L A Hahn, *Kgl Danske Videnskab Selskab Biol Medd* **15**, 5 (1940)
[138] C Entenman, I L Chaikoff, and H D Friedlander, *J Biol Chem* **162**, 111 (1946)
[139] M Beauvallet and S Manuel, *Compt rend soc biol* **144**, 1599 (1950)
[140] A E Porter, *Biochem J* **10**, 523 (1916)
[141] P Kyes, *Biochem Z* **4**, 99 (1907)
[142] C Delezenne and E Fourneau, *Bull soc chim France* **15**, 421 (1914)

characteristics of a globulin, whereas esterases resemble albumins [116] Lipases have been demonstrated in the digestive juices of the gastrointestinal tract, bacteria, and plants [117] Most of the so-called lipases described in blood and tissues appear to be esterases. The most important lipase in the small intestine is pancreatic lipase,[118] and it splits triglycerides into di- and monoglycerides and fatty acids, glycerol being slowly liberated [119] Bile salts are necessary for the activation of pancreatic lipase, and the environmental conditions have a marked effect on the rate of reaction and the nature of the end products.[120, 121]

d. Lecithins and Cephalins. (1) *Biosynthesis.* It has been assumed for some years that these two phospholipids are synthesized in the body, but conclusive proof had to await the effective labeling of various parts of the molecule. The incorporation of the fatty acids,[122] phosphate,[123, 124] and choline,[125] into the lecithin molecule in the tissues has been demonstrated The exact nature of the precursors is not yet known—there is some evidence to show that glycerophosphate,[126] glycerylphosphorylcholine[127] and lower glycerides[128] may play a part in lecithin synthesis under different circumstances. A lecitholipase which will synthesize glycerylphosphorylcholine has been shown to be present in cells [127] Phospholipid formation has been shown to be dependent upon oxidative activity in the cell, and synthesis rapidly declines under anaerobic conditions, or after poisoning with cyanide or other respiratc [illegible]

in brain slices [130] [illegible]

synthesis, which [illegible]

[116] D Glick and C G King, *J Am Chem Soc* **55**, 2445 (1933)

[117] W. R Bloor, Biochemistry of the Fatty Acids, Reinhold Publishing Corp , New York, 1943.

[118] C Bernard, Mémoire sur le pancréas et sur la rôle du suc pancréatique dans les phénomènes digestifs, particulièrement dans la digestion des matières grasses neutres, Baillière, Paris, 1856

[119] A C Frazer and H G Sammons, *Biochem J* **39**, 122 (1945)

[120] A. K Balls, M B Matlack, and I W Tucker, *J Biol Chem* **122**, 125 (1937)

[121] P Desnuelle, M Naudet and M J Constantin, *Biochim et Biophys Acta* **5**, 561 (1950), *ibid* **7**, 251 (1951)

[122] R G Sinclair, *J Biol Chem* **115**, 211 (1936)

[123] L A Hahn and G C Hevesy, *Biochem J.* **32**, 342 (1938)

[124] C Artom, G Sargana, and E Segrè, *Arch intern physiol* **47**, 245 (1938)

[illegible] (1945)

[illegible] 94, 131

(1952).

[129] A. Taurog, I L Chaikoff, and I Perlman, *J Biol Chem* **145**, 281 (1942)

[130] H Schachner, B A Fries, and I L Chaikoff, *J Biol Chem* **146**, 95 (1942)

enates. The cytoplasm of the cell appears to play more part than the nucleus[131, 132]

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[131] G C Hevesy, *Nature* **156**, 534 (1945)
[132] G. L Ada, *Biochem J* **45**, 422 (1944)
[133] M C Fishler, A Taurog, I Perlman, and I L Chaikoff, *J Biol Chem* **141**, 809 (1941)
[134] B A Fries, H Schachner, and I L Chaikoff, *J Biol Chem* **144**, 59 (1942)
[135] I L Chaikoff and D B Zilversmit, *Advances in Biol Med Physics* **1**, 321 (1948)
[136] E Chargaff, K B Olson, and P F Partington, *J Biol Chem* **134**, 505 (1940)
[137] G C Hevesy and L A Hahn, *Kgl Danske Videnskab Selskab Biol Medd* **15**, 5 (1940)
[138] C Entenman, I L Chaikoff, and H D Friedlander, *J Biol Chem* **162**, 111 (1946)
[139] M Beauvallet and S Manuel, *Compt rend soc biol* **144**, 1599 (1950).
[140] A E Porter, *Biochem J* **10**, 523 (1916)
[141] P Kyes, *Biochem Z* **4**, 99 (1907)
[142] C Delezenne and E Fourneau, *Bull soc chim France* **15**, 421 (1914)

venoms[143] Lecithinase C splits off choline only, forming a phosphatidic acid, while lecithinase D removes phosphorylcholine, leaving a diglyceride[144] This last type of lecithinase is found in *Cl. welchii* toxin, and lecithinases A, B, and D are said to occur in the intestinal mucosa, and possibly in other tissues[145, 146] Specific enzyme systems acting on cephalins have not been described, although lysocephalins are also formed by the action of the lecithinase A in snake venom[147]

e. Sphingomyelins and Cerebrosides. Little is known about the anabolic and catabolic reactions of these two closely related lipids. Sphingomyelins are not hydrolyzed by lipases, lecithinases, or alkaline phosphatases However, the acid-amide link can be broken enzymatically, and the phosphoric acid and choline can also be split off by suitable esterases. Radiophosphorus is incorporated more rapidly into sphingomyelins than into lecithins in the brain[136] A cerebrosidase in the pancreas has been described. In Niemann-Pick's disease sphingomyelins are greatly increased and collect in the reticulo-endothelial cells in most organs, while in Gaucher's disease cerebrosides accumulate in the spleen. In Tay-Sach's disease the normal galactose-containing cerebrosides are largely replaced by glucose-containing gangliosides There may be some obscure disturbance of the metabolism of these lipids in these rare conditions.[112, 148]

II. Tissue Lipids

Over thirty years ago, Terroine[149] divided biological lipids into two groups—an "élément constant" and an "élément variable"—the former remaining relatively unchanged with altering physiological conditions, while the latter showed wide variations Although, nowadays, we know that the tissues do not have a constant lipid composition in the strictest sense of this term, the fundamental importance of Terroine's broad generalization to the understanding of lipid metabolism remains. We may, therefore, consider the lipids of animal tissues in two main groups—those that appear to be primarily concerned in the structural organization of the tissues and those that are mainly used as a source of energy.

1 Structural Lipids the "Élément Constant" of Terroine

a. Alipemic Blood Plasma. (1) *Lipid Components.* Plasma collected from a fasting animal is normally a clear limpid fluid The lipids present are

[143] S Belfanti, *Z Immunitätsforsch* **66**, 449 (1928).
[144] M G MacFarlane and B C J G Knight, *Biochem J* **35**, 884 (1941)
[145] E. J. King, *Biochem J* **25**, 799 (1931), *ibid.* **28**, 476 (1934)
[146] E Kahane and J Levy, *Compt rend* **219**, 431 (1944)
[147] D. Fairbairn, *J Biol Chem* **157**, 633 (1945)
[148] B Ottenstein, G Schmidt, and S J Thannhauser, *Blood* **3**, 1250 (1948)
[149] E F Terroine, Contribution à la connaissance de la physiologie des substances grasses et lipoïdiques, Masson, Paris, 1919.

mainly phospholipids, cholesterol, and cholesterol esters. Practically no triglycerides are present, the phospholipids are lecithins and sphingomyelins, with small quantities of cephalins The fatty acids contained in the phospholipids are commonly palmitic, oleic, and linoleic The normal phospholipid level in man is in the region of 220 mg per 100 ml plasma The phospholipids in the plasma of different animals vary over a wide range and appear to be lowest in the guinea pig and the rabbit, (less than 50 mg per 100 ml), intermediate in horse, cow, pig, dog, and man, and highest in the eel [150, 151, 152] The proportion of phospholipid as cephalin is 3 to 8% in dog, ox, pig, and human serum, but may reach 20% in the turkey [153]

Over 60% of the cholesterol in plasma is esterified with fatty acids, which are usually more unsaturated than those found in the plasma phospholipids The normal total blood cholesterol level in man is in the region of 200 mg per 100 ml of plasma The amount of cholesterol appears to run fairly parallel to the phospholipid content, being lowest in the guinea pig (less than 50 mg per 100 ml) and highest in the eel [150] Striking differences in cholesterol levels have also been recorded in different strains of rats, which were attributable to more than one gene [154] It is claimed that only cholesterol occurs in ox plasma and that the plant sterols which are abundant in this animal's diet are not found in the blood [97] In the dog and other animals, however, as much as 25% of the unsaponifiable material is not cholesterol but a complex mixture of unknown composition [155]

(2) *Structural Organization* Although most of the lipid in the plasma is insoluble in water, 500 mg or more may be present in 100 ml of clear plasma Ether extraction of the lipid from natural plasma at normal temperatures is ineffective, but much more complete extraction occurs if alcohol is added to the ether,[156] or if the plasma is frozen to −25° [157] Separation of the plasma proteins by electrophoresis, ultracentrifugation, or precipitation methods indicates that lipids are mainly associated with the α- and β-globulin fractions Some 75% of the cholesterol and 85% of the phospholipid in the plasma is found to separate with these globulin fractions on electrophoresis [158] These characteristics appear to be due to the formation of molecular complexes between the lipids and plasma proteins Macheboeuf[159] prepared a lipoprotein complex from horse serum which was water-

[150] A Mayer and G Schaeffer, *J physiol path gén* **15**, 984 (1913)
[151] W R Bloor, *Bull soc chim biol* **3**, 451 (1921)
[152] W E Taylor and J M McKibbin, *J Biol Chem* **188**, 677 (1951).
[153] R G Sinclair, *J Biol Chem* **174**, 343 (1948)
[154] H I Kohn, *Am J Physiol* **163**, 410 (1950)
[155] R J Anderson, *J Biol Chem* **71**, 407 (1926).
[156] M Macheboeuf and G Sandor, *Bull soc chim biol* **14**, 1168 (1932)
[157] A S McFarlane, *Nature* **149**, 349 (1942)
[158] G Blix, A Tiselius, and H Svensson, *J Biol Chem* **137**, 485 (1941).
[159] M Macheboeuf, *Bull soc chim biol* **11**, 268, 483 (1929)

soluble and contained 40% of lipid, consisting largely of lecithin and cholesterol

More recently Cohn and his collaborators[160] have used alcohol precipitation methods to separate two lipoproteins from human plasma The α-lipoprotein is soluble in salt-free water, represents 3% of total plasma protein, and contains 35% of lipid and 65% of amino acid residues. It is 300 Å. long and 50 Å wide, and its molecular weight is of the order of 200,000 It appears to resemble the lipoprotein described by Macheboeuf. The β-lipoprotein is spherical, with a diameter of 185 Å., and is soluble in dilute salt solutions. It represents about 5% of the total protein and contains 75% of lipid, of which 30% is phospholipid, 45% cholesterol, and 25% protein. Most of the sterols and all the carotenoids in blood plasma are associated with β-lipoprotein. The anhydrous molecular weight is estimated to be 1,300,000. β-Lipoprotein accounts for three-quarters of the lipid of fasting human plasma and strongly resembles the so-called X-protein.[161, 162]

The thromboplastic material which can be prepared from lungs and many other tissues appears to be a lipoprotein, containing cephalin.[163, 164] Heparin splits off the phosphatide from the lipoprotein complex[165]

Evidence has been put forward to suggest[166, 167] that heparin may play an important part in the accommodation of lipids, including glycerides, in apparently alipemic plasma The relationship of this effect to lipoprotein structure is still obscure and the action of heparin will be discussed in relation to alimentary hyperlipemia. Lipid associations with protein and carbohydrate appear to play an important part in many antigen-antibody reactions. A phosphatidic acid, cardiolipid, has been isolated from beef heart muscle and acts as an antigen in the Wasserman reaction.[168]

The importance of lecithin in normal plasma structure is indicated by the disruptive effect that the D-lecithinase of *Cl. welchii* toxin has upon it. If human plasma is incubated with this enzyme, turbidity develops as the phospholipid level falls, owing to the liberation of diglyceride and the precipitation of a proteinous deposit. The reaction can be prevented by the specific antitoxin These changes can be observed in a sample of plasma containing no triglyceride, and the diglyceride that accumulates is one of

160 F R N. Gurd, J L Oncley, J T Edsall, and E J Cohn, *Discussions Faraday Soc.* **6**, 70 (1949)

161 A S McFarlane, *Biochem J* **29**, 407 (1935)

162 K O Pedersen, Ultracentrifugal Studies on Serum and Serum Fractions, Almquist & Wiksells, Uppsala, 1945

163 E. Chargaff, F W. Bancroft, and M S Brown, *J. Biol Chem* **116**, 237 (1936)

164 S S Cohen and E Chargaff, *J Biol. Chem* **136**, 243 (1940).

165 [illegible] (1940).

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the two end products of the lecithinase reaction, the other being phosphorylcholine D-Lecithinase has no visible effect on guinea pig plasma The proportion of phospholipid to cholesterol has been thought to be a factor in maintaining proper lipid dispersion. Some disturbance of the relationship between these two lipids might be a factor in the deposition of cholesterol in blood vessel walls in atherosclerosis Lecithin may have some relationship to the structure of the chylomicron, which will be discussed later[169, 170, 171]

The blood cholesterol tends to increase slowly with age, but no satisfactory correlation between dietary intake and blood cholesterol has yet been established[172] Recently, Gofman and his collaborators have demonstrated[173] *the presence of peculiar macromolecules by ultracentrifugal studies of* human sera which appear to correspond to similar lipoproteins observed in the blood of hypercholesterolemic, but not of normal, rabbits The percentage of subjects with these Sf10–20 molecules increases with age, and they are found in the blood of 50% of people over 40 They also occur in 90% of patients suffering from severe degenerative disease of the cardiovascular system It is claimed that the formation of these abnormal lipoproteins can be prevented by restriction of dietary cholesterol or by the administration of heparin[174] Whether these unusual components really represent an unstable form of lipid that may, under certain circumstances, be deposited in the blood vessel walls must await further proof

b. General Cell Structure. (1) *Lipid Components* Cytoplasm of all cells contains about 1 to 2% of lipids, the other main components being 85 to 90% of water, 7 to 10% of protein, and 2 to 3% of other organic and inorganic material The approximate molecular proportions of water, lipid and protein in protoplasm are 20,000 10.1 The lipids present in cytoplasm are mainly phospholipids, cerebrosides, and cholesterol Glycerides do not appear to form an important part of the structure of most cells.

(2) *Structural Organization of Cell Cytoplasm* As in the plasma, the lipids concerned in cell structure are closely associated with proteins A number of important lipoproteins and proteolipids have been isolated recently from various tissues Thus, plasmalogen (acetal phosphatide), which can be demonstrated *in situ* in cell cytoplasm, has been isolated and identified[175]

[171] F P O Nagler, *Brit J Exptl Path* 20, 473 (1939)

[172] A C Frazer, J J Elkes, H C Sammons, A D T Govan, and W T Cooke, *Lancet* April 14, 457 (1945)

[171] E H Ahrens, Jr and H G Kunkel, *J Exptl Med* 90, 409 (1949)

[172] A Keys, O Mickelsen, E v O Miller, E R Hayes, and R L Todd, *J Clin Invest* 29, 1347 (1950)

[173] J W Gofman, H B Jones, F T Lindgren, T P Lyon, H A Elliott, and B Strisower, *Circulation* 2, 161 (1950)

[174] D M Graham, T P Lyon, J W Gofman, H B Jones, A. Yankley, J Simonton, and S White, *Circulation* 4, 666 (1951)

[175] S J Thannhauser, N F Boncoddo, and G Schmidt, *J. Biol Chem* 188, 417, 423, 427 (1951)

Proteolipids have been isolated in crystalline form from brain and other tissues They are insoluble in water but freely soluble in chloroform-methanol-water mixtures. They consist of various proportions of protein, phospholipids, and cerebrosides.[176] A water-soluble and electrophoretically homogeneous lipoprotein, strandin, with a molecular weight of at least 250,000, has been isolated from brain, but only traces have been found in other tissues [177] These complexes of protein and lipids may be important constituents of the ground substance of protoplasm.

It has been known for many years that the cytoplasm contains structural elements such as *mitochondria, and the Golgi apparatus On histochemical* grounds both these structures have been considered to contain phospholipid, which, from their staining reactions, was thought to be closely associated with protein. The development of modern cell fractionation techniques[178] has made it possible to isolate and analyze specimens of mitochondria and other particulate material, such as the microsomes, which are minute submicroscopic particles, about 500 to 3000 Å. in diameter, present in hyaloplasm.[179] The microsomes have been shown to contain lipids associated with ribonucleoprotein Analysis shows 2% of inositol and 40 to 45% of lipids, of which 75% is phospholipid. The fatty acids are highly unsaturated and in mouse liver cells may contain as much as 20% of tetraenoic acid, probably arachidonic acid Cytochrome *c* has been found to be associated with the microsomes.[180, 181]

The staining reactions of the mitochondria were thought to indicate a high lipid content. Frozen and dried liver cells were extracted for long periods with fat solvents, however, without alteration in staining properties, and there were indications that the major component might be protein.[182] This was fully substantiated by analyses of mitochondria separated by centrifugation. The mitochondria from hepatic cells appear to contain from 65 to 75% of protein and 25 to 35% of lipid, a high proportion of which is phospholipid. From the difficulty found in extracting the lipid, it would appear to be firmly bound to the protein component.[183] The polarizing optics of mitochondria suggest that the protein is arranged parallel to the axis, while the lipid is placed at right angles to this plane [184] The presence of mitochondria and stainable fat appears to have a reciprocal relationship in some cells. The mitochondria are associated with the greater

176 J Folch and M Lees, *J. Biol Chem* 191, 807 (1951)

177 [illegible]

178

179

180

181 N Kretchmer and C. [illegible] Barnum, *Arch Biochem* [illegible] (1951)

182 R R Bensley and I Gersh, *Anat Record* 57, 207, 217, 369 (1933).

183 R. R Bensley, *Anat. Record* 69, 341 (1937)

184 C Grave, *Anat Record (Suppl)* 70, 85 (19

part of the cytochrome oxidase and succinic dehydrogenase in the cell cytoplasm They also contain vitamin C and glutathione.[185, 186]

The Golgi organ appears as a stainable reticulated structure in many differentiated cells, but its existence in this form in living cells is still the subject of controversy. Although the Golgi apparatus can be displaced in the cell by high-speed ultracentrifugation, satisfactory analyses of separated material have not yet been reported Recent histochemical studies support the view that phospholipids, probably lecithins and cephalins, are present in the Golgi elements The reticulated appearance of the Golgi apparatus has been attributed to myelin figures produced in cells during fixation Whether the appearance of the Golgi apparatus in fixed cells is an artefact or not, it seems clear that the part of the cell in which it occurs has a high phospholipid content [187, 188, 189]

(3) *Cell Membrane* Many cells appear to have a fairly well-defined membrane which surrounds the cell and may connect with strands of material running through the substance of the cell, forming a cytoskeleton Such cell membranes, and probably other forms of organization of the cell border, appear to depend for their structure largely upon the presence of lipids and proteins, although carbohydrates and other substances may also play a part This membrane plays a vitally important part in the life of the cell, since its structure and molecular composition decide the materials which can pass from the environment into the cell and vice versa That lipids are an important factor in membrane structure is suggested by the fact that lipid solubility plays an important part in the penetration of molecules into cells and that there is a high electrical impedance at the membrane However, since water and water-soluble molecules can also pass through these membranes and the interfacial tension at the cell surface is far less than that found at an oil-water interface, it is thought that protein, as well as lipid, must be involved in the surface layers It has been further suggested that cell membranes may be a tricomplex involving proteins, lipids, and cations [190]

The most extensively studied mammalian cell membrane is that of the erythrocyte Preparations of these cell membranes can be made, but it is impossible to say how much of the structure is lost in this process, or how great the contamination with cytoplasmic material may be It is probable that the structure of the cell membrane merges almost imperceptibly into

[185] G H Hogeboom, A Claude, and R D Hotchkiss, *J Biol Chem* 165, 615 (1946)

[186] G Bourne, Cytology and Cell Physiology, Oxford University Press, London, 1942

[187] H W Beams, *Biol Symposia* 10, 71 (1943)

[188] J R Baker, *Quart J Microscop Sci* 85, 1 (1944)

[189] A Claude and G E Palade, *J Morphology* 85, 35, 71 (1949).

[190] H L Booij, *Discussions Faraday Soc.* 6, 143 (1949)

that of the cytoplasm. If this cell membrane is examined, it is found to contain 3.4% of the total cell solids The lipids present are cholesterol and phospholipids—largely cephalins and sphingomyelin. The thickness of the membrane, not allowing for water content, is 190 Å. in man, 250 Å. in the dog, 125 Å. in the rabbit, 120 Å. in the sheep, and 75 Å. in the ox. The total lipid in these cells would provide a continuous layer of lipid 31 Å. thick in man, and 38 Å , 33 Å., and 32 Å., respectively, in dog, sheep, and rabbit This would just allow the formation of a bimolecular layer. It would not be possible to form a monolayer of cholesterol molecules alone round any of the red cells, with the possible exception of the ox, which has a lipid/cholesterol ratio of 1 5/1 as compared with 5/1 in man. The amount of protein present would give a continuous surface layer 37 Å thick and of density 1 3 g cm^{-3}.[191] The protein, stromatin, is related to the collagens [192] Some 40 to 60% of the lipids are not extractable with ether and are thought to be bound to the protein as secondary valency compounds.[193] The polarization optics of the red cell membrane support the view that the lipid molecules are arranged radially and the protein molecules tangentially.[194] The electrical mobility measurements and electron microscope studies of the red cell membrane leave little doubt that the surface has a mosaic structure with lipid molecules embedded in a layer of protein, but the lipid molecules are not sufficiently close together to constitute a homogeneous outer layer.[195, 196]

If red blood cells are placed in hypotonic salt solution, hemoglobin escapes and sodium ions may begin to leak into the cell. This process of hemolysis continues until only the "ghost" of the red cell remains, consisting of the cell membrane and a variable amount of cytoplasmic residue If during the hemolytic process concentrated salt solution is added, escape of hemoglobin ceases and the integrity of the membrane is, at least partly, restored [197, 198, 199] This type of hemolysis is considered to be due to rupture of the cell membrane Hemolysis can also be caused by a number of lipid solvents and surface active agents, which can be shown to penetrate into

[191] E Ponder, *Discussions Faraday Soc* 6, 152 (1949)

[192] E Jorpes, *Biochem J* 26, 1488 (1932)

[193] A K Parpart and A J Dziemian, *Cold Spring Harbor Symposia Quant Biol* 8, 17 (1940)

[194] F O Schmitt, R S Bear, and E Ponder, *J Cell Comp Physiol* 9, 89 (1936), 11, 309 (1938)

[195] R F Furchgott and E Ponder, *J Exptl Biol* 17, 117 (1940), *J Gen Physiol* 24, 447 (1941)

[illegible]

[illegible] nschrift, [illegible]

[198] L E. Bayliss, [illegible]

[199] H Davson and E Ponder, *Biochem J.* 32, 756 (1938)

cholesterol-containing surface films.[200] The "ghosts" obtained from this type of hemolysis are different from those produced by hypertonic salt, since hemolysis cannot be reversed with these cells by the addition of concentrated salt solution Study of the behavior and surface characteristics of these "ghosts" suggests that the lytic agents have removed or displaced lipid molecules from the cell membrane, so that numerous small holes develop, in contrast to the single rupture in hypotonic "ghosts."[201, 202] This agrees with the appearances of the membrane in electron micrographs after treatment with lipid solvents [196]

c. Nervous Tissue. (1) *Lipid Components* Lipids play an important part in the structure of nervous tissue, the most important components being phospholipids, cholesterol, and cerebrosides The proportion of these lipids varies in the axoplasm and in myelin In mammalian nerves, the proportions in the axoplasm are of the order of 4 parts of phospholipid to 1 part each of cerebrosides and cholesterol, whereas in myelin the proportions are 2:1:1 Lecithin and cephalin are the main phospholipids of axoplasm, whereas myelin has a much greater proportion of cephalins and sphingomyelins Cholesterol esters are absent from normal myelin The absolute quantities of lipids are also different in these two parts of the neurone Differences in the lipid content of white or gray matter, or various peripheral nerves, can probably be attributed to the relative proportions of myelin, axoplasm, and structural tissue present The lipid content of mammalian white matter is usually in the region of 60% of the dry weight, the phospholipids forming about 30% and cholesterol and cerebrosides approximately 15% each, whereas in the gray matter the total lipids are of the order of 30%, of which phospholipids form about 20%, and cholesterol and cerebrosides about 5% each In peripheral myelinated nerve, the total lipids may be in the region of 50% of dry weight, the phospholipids forming about 25%, whereas the cholesterol and cerebrosides form approximately 12% each Non-myelinated nerves, on the other hand, contain only about 15% of lipids, of which 10% is phospholipid, and the cholesterol and cerebrosides are each of the order of 2 5% [203, 204] Relatively little difference is found between the white and gray matter of the brain in infants As myelination proceeds there is a great increase of sphingomyelins, cerebrosides, and cholesterol in the white matter [205]

Cerebrosides occur at levels between 5 and 15% of dry weight in mam-

[200] J H Schulman and E. K Rideal, *Proc Roy Soc* (*London*) **B122**, 29, 46 (1937)
[201] H Fricke and H. J Curtis, *J Gen Physiol* **18**, 821 (1935)
[202] E Ponder and D Marsland, *J Gen Physiol* **19**, 35 (1935)
[203] J L W Thudichum, Die chemische Konstitution des Gehirns, des Menschen, und der Tiere, Pietzcker, Tübingen, 1901
[204] G Brante, *Acta Physiol Scand* **18**, *Suppl* 63 (1949)
[205] A C Johnson, A R McNabb, and R J Rossiter, *Biochem J*, **44**, 494 (1949)

malian brain, being highest in the white matter; the over-all cerebroside content of turtle and hen brain is in the region of 6%, and only 3 to 4% is found in the brain of fishes and amphibia. Cerebrosides appear to be absent from the brain of the octopus[206] and from the giant axon of the squid [207] The nervous system of the lobster contains some 80% of a lecithin-like phospholipid, but no cerebrosides.[204]

The fatty acids in the brain lipids are found to have a high molecular weight and a high degree of unsaturation The high molecular weight may be largely attributed to the presence of the saturated 24-carbon lignoceric acid and closely related hydroxy- and monoethenoid fatty acids, which, with stearic acid, constitute the main fatty acids found in cerebrosides and sphingomyelins. Polyethenoid acids do not occur in any of the brain lipids except lecithin and possibly phosphatidylethanolamine, so that the arachidonic acid and other highly unsaturated fatty acids found in brain lipids must be associated with these fractions If young growing rats are deprived of unsaturated fatty acids, the tetraenoic acid content of the brain lipids may be reduced by 15 to 25%. The total phospholipid is not altered, and the tetraenoic acids are replaced by other less unsaturated acids [139]

(2) *Wallerian Degeneration.* In Wallerian degeneration of mammalian peripheral nerve, marked changes in lipid composition and structure are observed For 8 days after section little change is seen except a decrease of neutral fat, which is probably in the associated connective tissue, and an increase in the wet weight of the nerve. From the eighth day onwards the *myelin lipids, cerebrosides, sphingomyelins, and free cholesterol show* a rapid decrease and cholesterol esters appear. Cephalin also decreases rapidly, but lecithin much more slowly. The products of lipid breakdown are removed by phagocytes When the nerve is crushed instead of being sectioned, the disappearance of the lipids follows a similar course, but as regeneration occurs the myelin lipids gradually return, although they only reach 44% of the level of the controls in 144 days. Ester cholesterol, which forms in the nerve during the degenerating period, completely disappears, but the lecithin, which falls slowly, shows no signs of increasing again by the 144th day In the central nervous system demyelination appears to be accompanied by similar chemical changes, with liberation of choline from sphingomyelin as a characteristic feature Triglyceride formation is not observed during degeneration or regeneration [204, 208, 209]

(2) *Structural Organization of Myelin* The lipids in the brain are probably present as lipoprotein units, and, as already described, a number of macro-

206 [illegible] 120 (1938)
207
208 [illegible] 45, 500 (1949)
209 [illegible] 18 (1950)

molecules have been isolated The most important ordered structure in nervous tissue, however, is myelin This material has been extensively studied with the polarizing microscope, X-ray diffraction techniques, and the electron microscope The effects of tension, drying, and lipid solvents on myelin structure have been examined in considerable detail These various studies indicate that myelin is probably a lipoprotein unit, with the lipid molecules arranged radially in relation to the axis cylinder and the protein tangentially The unit cell of this system in fresh nerve is of the order of 170 Å long, which might be formed by two bimolecular leaflets of lipid which are separated from each other by a sheet of protein Water plays an important part in the structure of myelin When the nerve is dried, considerable modification occurs in the X-ray diffraction spacings, these are reversible in the early stages, but a point is eventually reached at which irreversible changes in the diffraction pattern are seen, possibly indicating separation of individual lipid components It is interesting that conduction along the nerve appears to cease at the same time that the irreversible changes occur in the myelin [210-213]

d. Adrenals and Gonads. The adrenal cortex contains a high proportion of cholesterol, cholesterol esters, free fatty acids, and phospholipids, but no glycerides [214] The fatty acids are highly unsaturated and include arachidonic acid [215] In rabbits, however, it has been claimed that only phospholipids and cerebrosides are present, unless cholesterol is fed [216] Great differences in the cholesterol present in various species have been reported—0 5% in cow and sheep, 5% in guinea pig, and 10% in cat [217] ACTH is said to increase the turnover of sterol hormones from the adrenal, with a consequent fall in plasma cholesterol [218] Adrenalectomy does not cause consistent changes in blood cholesterol [219, 220]

Phospholipid, cerebrosides, and cholesterol are found in the gonads. Changes in lipid content occur during the sexual cycle in the ovaries of sows, rabbits, and frogs, but not of guinea pigs The phospholipids and

[210] F O Schmitt and K J Palmer, *Cold Spring Harbor Symposia Quant Biol* **8**, 94 (1940)

[211] F O Schmitt, R S Bear and K J Palmer, *J Cell Comp Physiol* **18**, 31 (1941)

[212] J J Elkes and J B Finean, *Discussions Faraday Soc* **6**, 134 (1949)

[213] G Rozsa, C Morgan, A Szent Györgyi, and R W. G Wyckoff, *Biochim. et Biophys Acta* **6**, 13 (1950)

[214] N C Borberg, *Skand Arch Physiol* **32**, 287 (1915)

[215] W C Ault and J B Brown, *J Biol Chem* **107**, 607 (1934)

[216] R Bär and R Jaffe, *Z Konstitutionslehre* **10**, 321 (1925)

[217] C I Parhon and M Cahane, *Compt rend soc biol* **107**, 836 (1931)

[218] J W Conn, W C Vogel, L H Louis, and S S Fajans, *J Lab Clin Med* **35**, 504 (1950)

[219] E J Bauman and O M Holly, *J Biol Chem* **65**, 457 (1923).

[220] F S Randles and A Knudson, *J Biol Chem* **76**, 89 (1928)

free cholesterol reach a maximal level at the time of greatest activity of the corpus luteum [221, 222, 223]

e. Muscle. Apart from glycerides, which may form adipose tissue closely associated with muscle fibers, lipids appear to play some part in muscle structure, mainly phospholipid and cholesterol being concerned. In all species the phospholipid content is highest in cardiac muscle, while cholesterol is highest in plain muscle. In man the heart contains about 7% of dry weight as phospholipid, 4.5% being lecithin, 2% cephalin, and the remainder sphingomyelin The ratio of phospholipid to cholesterol is about 10 to 15:1 in heart and striped muscle and 5:1 in plain muscle The usual fatty acids are present In heart muscle phospholipid is fairly constant throughout a wide range of species at 6 to 7%, but cholesterol varies greatly, being 0.5% in mammals but increasing in cold-blooded animals up to 4% in the alligator and the turtle. Striped muscle shows a phospholipid level of about 4 5% in all species, and the cholesterol remains much the same at about 0.25 to 0 3%. In smooth muscle the phospholipid is about 3% and the cholesterol about 0 75 to 1 0%. There may be some relationship to activity—the pectoralis muscle of the bat has an exceptionally high phospholipid content of 8%.[224]

2. Lipids Primarily Utilized for Energy: the "Élément Variable" of Terroine

The main variable lipid component of animal tissues is triglyceride. This is the form in which lipids are mainly ingested, stored, and perhaps metabolized, in almost all animals—the storage of waxes instead of glycerides being the major exception The amount of these fats present in the body is dependent upon an adequate food supply, and during starvation glyceride levels in the body fall extremely low Lecithins appear to play a part in the utilization of glycerides for energy purposes, as well as being structural lipids—different types of lecithin molecules being involved

a. Dietary Lipid. Lipids in the diet fall naturally into two parts—animal and vegetable The young mammal consumes milk, and the adult carnivore or omnivore eats animal lipids, man and domestic animals may also ingest dairy products in adult life; vegetable lipids are contained in the diet of omnivorous and herbivorous animals The lipids of the average mixed diet of a man in Great Britain are about 90% animal and 10% vegetable in origin, the proportion of vegetable lipid in the human diet is much greater in some other countries.

[221] A. Chauffard, G Laroche ,and A Grigaut, *Compt rend soc biol* **72**, 223, 265 (1912).
[222] W. R Bloor, R Okey, and G W. Corner, *J Biol Chem* **86**, 291 (1930)
[223] E M Boyd, *J Biol Chem.* **108**, 607 (1935), *ibid* **112**, 591 (1936), *J Physiol* **91**, 394 (1938)
[224] W. R Bloor, *J. Biol Chem* **114**, 639 (1936)

The vegetable fats are mainly glycerides, but some interesting differences in fatty acid composition occur. Most leaves contain about 5% of lipids, and a common feature is the presence of quite considerable amounts of linolenic acid. This is particularly the case in grass fats and several green vegetables. Good pasture grass contains about 5% lipid, of which 1 to 2% is glyceride, 1% unsaponifiable matter, and 0.3% phosphatides. Cholesterol does not occur in plants, but other sterols may be present. The fatty acids consist of 10% saturated and 90% unsaturated, oleic, linoleic, and linolenic acids being present in the proportions 1:2:3.[225] Many seed oils are also particularly rich in linolenic acid. Root fats usually contain mainly oleic acid—the mangel has 7% of lipids.[226] Fruit coat fats, such as the palm oils and olive oil, contain oleic, linoleic, and palmitic acids. Cereals commonly contain about 10% of lipid in the germ and 1% in the endosperm, in rice there may be as much as 35% of lipid in the germ and 10% in the endosperm. Linolenic acid occurs in wheat, millet, rye, and barley seed fats and also in wheat germ. Aquatic flora show a fairly high proportion of unsaturated fatty acids in their lipids. C_{16} acids are the most important in the green algae, while the C_{20} and longer acids form a more important part of the brown and red algae. The marine diatom has a high content of C_{16} acids.[227] Waxes are not commonly ingested in any quantity, but the wax moth (*Galleria mellonella*) has been shown to utilize 50% of the wax it consumes.[228]

b. Intestinal Cell. Although absorption has been studied in a general way in a number of different animals and in human subjects, most of the available information on the cellular phase of absorption is derived from studies in rats. The intestinal cells, which are the absorbing units in the small intestine, appear to have the same structure in several different species.[229]

(1) *Lipid Components.* In the fasting rat, or after feeding protein or carbohydrate, the intestinal cells contain no stainable lipid and on analysis show the small quantities of cholesterol and phospholipid found in all tissues. The cells may become loaded with lipids, however, if these substances are fed to the animal. On a normal mixed diet an increase of glycerides, fatty acids, cholesterol, and phospholipids may be demonstrated, and these various lipid components can be visualized in the cell by appropriate histochemical methods.

(2) *Intraluminar Phase of Fat Absorption.* Triglyceride fats must undergo certain changes in the intestinal lumen preparatory to absorption. For

[225] J. A. B. Smith and A. C. Chibnall, *Biochem. J.* 26, 218, 1345 (1932).
[226] A. Neville, *J. Chem. Soc.* 101, 1101 (1912).
[227] J. A. Lovern, *Biochem. J.* 30, 387 (1936).
[228] W. Niemierko and P. Wlodawer, *Acta Biol. Exptl.* (*Warsaw*) 15, 69 (1950).
[229] J. R. Baker, *Quart. J. Microscop. Sci.* 84, 73 (1942).

many years it was thought that complete hydrolysis to fatty acid and glycerol was an essential step in fat absorption[113, 117] It has been now shown, however, that hydrolysis of *glycerides by pancreatic lipase is only* partial and that fats can be absorbed both as fatty acids and as glycerides[129, 230-231] The products of pancreatic lipolysis are diglycerides, monoglycerides, fatty acids, and glycerol.[119] These lower glycerides and fatty acids, with bile salts, form an effective emulsifying system which, even under the acid conditions prevailing in the intestine, disperses the glyceride fraction into particles of less than 0 5 μ in diameter.[235] Some of the fatty acids may be removed from the oil into the water phase—this occurs rapidly and easily with water-soluble butyric acid, but it is much more difficult to achieve with longer chain fats Thus the dietary fat is presented to the intestinal cell for absorption in two forms—as water-soluble molecularly dispersed fatty acids, or as small particles of water-insoluble material consisting of glycerides, fatty acids, and other oil-soluble materials

(3) *Cellular Phase of Absorption.* The intestinal cells have an outer "brush border" which is pierced by fine canals running at right angles to the surface These canals are filled with lipid-staining material during fat absorption, but not during protein or carbohydrate absorption.[236] The size of the canals is such that particles rather less than 0 5 μ could enter them. Recent studies using the electron microscope show that the outer border may have a fine fibrillar structure, but this is not incompatible with the existence of fine canals running in the same direction as these fibrils. Fat absorption is defective if bile or pancreatic juice are excluded from the intestine, when fine emulsification does not occur.[237] Paraffin is not hydrolyzed, so it is neither emulsified nor absorbed if given alone If it is introduced as a finely divided emulsion with a particle size of less than 0.5 μ, the paraffin particles pass into the intestinal cells.[235] If paraffin is mixed with an equal quantity of olive oil, fine emulsification and absorption of the mixture occurs[233] Thus, the fine emulsification of the glyceride residue appears to be an important step in the absorption of the water-insoluble fat fraction

[230] A. C Frazer, *Analyst* 63, 308 (1938)
[231] A C Frazer, Modern Trends in Gastroenterology, Butterworth, London, 1952, p. 477
[232] [illegible]
[233] [illegible] *Chim Acta* 34, 1641 (1951).
[234] [illegible]
[235] [illegible] *Physiol.* 103, 306 (1944)
[236] [illegible]
[237] [illegible] es, Butterworth, London, 1951, p 528
[238] J W Daniel, A C Frazer, J M French, and H G Sammons, *J. Physiol* 114, 26P, 1951.

Fat enters the cell border as a fine particle of glyceride and fatty acid. This glyceride is converted, at least in part, into phospholipid, lecithin leaving the intestine during fat absorption has been shown to be derived from dietary triglyceride.[122] It would, of course, be an interesting transport mechanism if every molecule of fat passes through this phospholipid stage, but the phosphorus turnover studies of Chaikoff and his colleagues[239] are said to exclude this possibility Whether the method used would show up intracellular phosphorus turnover of the kind suggested is, perhaps, open to doubt There is some[240] evidence that phospholipid does facilitate absorption, but its precise role in the mechanism is still obscure. The fat accumulates in the cell as fine particles provided that reasonable quantities of fat, say 250 mg per 100 g of body weight in the rat, are fed If grossly excessive amounts of fat are administered, the particles coalesce and the cell is seen to be loaded with fat globules [241] This appearance is commonly regarded as the "normal" picture of the intestinal cell during fat absorption.

The intestinal cells are capable of considerable metabolic activity, so that rearrangement of the glycerides might be anticipated The final demonstrable end products are phospholipids and triglycerides,[231] which will differ from the original dietary glycerides, since short-chain acids have been eliminated and other modifications of the fatty acids may have occurred Most of the cholesterol in the diet will probably be associated with the glyceride fraction The soluble fatty acid is difficult to trace—it presumably takes part in the various metabolic activities of the cell or passes out of the cell with other water-soluble substances

(4) *Distributive Phase of Fat Absorption* Negatively charged particles introduced into the tissues tend to pass into the lymphatics rather than the blood capillaries. The fine fat particles in the intestinal villi are no exception They pass into the lacteals and so find their way into the chyle and thence into the blood stream The particulate glyceride fraction and fatty acids and cholesterol dissolved in it pass by this route [242, 243, 244] Glycerides containing short-chain fatty acids,[245] or the fatty acids themselves of 10-carbon chain length or less,[246] are not found in the chyle They

[239] D B Zilversmit, C Entenman, and I L Chaikoff, *J Biol Chem* **172**, 637 (1948)

[240] V Auger, H S Rollman, and H J Deuel, Jr, *J Nutrition* **33**, 177 (1947)

[241] A C Frazer, *Arch sci physiol* **2**, 15 (1948)

[242] B Bloom I L Chaikoff, W O Reinhardt, C Entenman, and W G Dauben, *J Biol Chem* **184**, 1 (1950)

[243] J F Mead, L R Bennett, A B Decker, and M D Schoenberg, *Atomic Energy Project Report*, UCLA 88, 1950, p 596

[244] M W Biggs, M Friedman, and S O Byers, *Proc Soc Exptl Biol Med* **78**, 641 (1951)

[245] R H Hughes and E J Wimmer, *J Biol Chem* **108**, 141 (1935)

[246] B Bloom, I L Chaikoff, and W O Reinhardt, *Am J Physiol* **166**, 451 (1951)

must either be metabolized in the intestinal cell, or pass into the body by some other route. There is evidence to indicate that some of this fatty acid may pass to the liver by the portal vein.[247, 247a]

Dietary phospholipid is not hydrolyzed by intestinal lipases or phosphatases, and no active lecithinases can be demonstrated in the intestinal lumen in rats or in man.[248] The unhydrolyzed lecithin passes freely into the intestinal cell, and an extract of rat's intestinal mucosa can be shown to contain lecithinase. Cholesterol can be freely absorbed only in the presence of fat in most animals, except the rabbit, but the addition of extra cholesterol to dietary fat may cause some interference with fat absorption.[249] It is claimed that plant sterols are not absorbed, but there is considerable disagreement on this point among different workers. Labeled cholesterol has been shown to pass almost exclusively by the lymphatic route.[244] Most animals show considerable tolerance to a large increase of dietary triglycerides; guinea pigs, however, do not tolerate glycerides except in small quantities. The absorption of short-chain fatty acids has been extensively studied in the ruminant, and they are found to pass directly into the blood stream.[250]

c. Adipose Tissue. (1) *Lipid Components.* The essential lipid component of the adipose tissue of mammals is triglyceride, largely of palmitic and oleic acids. In ruminants there tends to be a definite increase of the amount of stearic acid present. An appreciable amount of linolenic acid occurs in the fat depots of the wild rabbit, and also of the horse, being probably derived from grass.[1] It is, however, absent from the depot fats of grass-fed ruminants, since the linolenic acid is hydrogenated in the rumen.[50] In the adipose tissue of marine animals there is a great increase of unsaturated long-chain C_{16-22} acids and the palmitic acid content of the glycerides is in the region of 10%. In many cold-blooded animals oleic tends to be replaced by palmitoleic acid. In fresh-water fish there is a preponderance of C_{16-18} acids, and unsaturated C_{16} acids may form 30% of the fatty acids in the glycerides. In sea-water fish, on the other hand, there are more of C_{18-22} acids. These differences between fresh-water and sea-water fish are found throughout the species,[251] with the exception of the sturgeon,[252] which has the fresh-water type of fat. The salmon, during the course of its development, changes the fatty acid pattern of its lipids in conformity with its passage to the sea.[253] Peculiar fatty acids occur in the depots of some fish,

[247] A. C. Frazer, *J. Physiol.* 102, 306, 329 (1943).
[247a] J. Y. Kiyasu, B. Bloom and I. L. Chaikoff, *J. Biol. Chem.* 199, 415 (1953).
[248] A. C. Frazer, P. E. Sagrott, and H. G. Sammons, *Proc. 1st Intern. Congr. Biochem.*, 1949, p. 596.
[249] R. P. Cook and R. O. Thompson, *Biochem. J.* 49, 72 (1951).
[250] [illegible]
[251]
[252]
[253]

the most striking being the presence of isovaleric acid in the porpoise and the dolphin,[254] and the presence of hydroxy fatty acids in the castor-oil fish.[255] The fat depots of amphibia and reptiles are, generally speaking, intermediate between those of fish and land animals. The adipose tissue of the green turtle is peculiar in that it contains high proportions of lauric and myristic acid.[256] The fat depots of sea birds that live on fish are of the marine type, in contrast to land birds.[257] The type of fat stored appears to be affected by the temperature of the tissue. In cold-blooded animals the fats tend to be more unsaturated and the monoethenoid acids are of shorter chain length—palmitoleic instead of oleic. In warm-blooded animals the lower melting point fats are found nearer the surface, the fat from deeper tissues having a higher melting point.[258] In the sperm whale family the fats in the depots are not glycerides, but mainly esters of long-chain fatty alcohols and highly unsaturated long-chain acids.[259] Similar esters are sometimes encountered in other fish.

The fatty acid composition of the fat depots closely resembles the dietary fat, provided that the fat intake is sufficiently high. On a low fat intake the dilution by fat derived from other sources becomes considerable. Small differences in iodine value, melting point, and other properties between dietary fat and absorbed fat in the chyle or fat depots can be readily explained. It has already been shown that short-chain fatty acids are removed from the glyceride fraction, it is possible that some of the unsaturated fats may also be removed, and in some animals they may be hydrogenated, the chyle frequently contains fat that has not just been absorbed but has been mobilized from elsewhere. Taking all these points into consideration, it is surprising that such a close correlation between dietary, chyle, and depot fat has so often been shown. The melting point and other characteristics of the depot fat of pigs and other animals can be controlled by balancing up the type of fat fed to the animal against the level of carbohydrate intake, since carbohydrates tend to make hard fats.[260] The fat depots of the eel were also found to resemble the dietary fat closely, when the fat intake was high.[261]

(2) *Characteristics of Adipose Tissue.* Fat is deposited in characteristic cells which have a more active metabolism than is commonly supposed. These cells are able to synthesize, assimilate, store, and mobilize glycerides

[254] M. E. Chevreul, Recherches chimiques sur les corps gras, 1823.
[255] W. M. Cox and E. E. Reiser, *J. Am. Chem. Soc.* **54**, 220 (1932).
[256] T. G. Green and T. P. Hilditch, *Biochem. J.* **32**, 681 (1935).
[257] J. A. Lovern, *Biochem. J.* **32**, 2142 (1938).
[258] A. Henriques and C. Hansen, *Skand. Arch. Physiol.* **11**, 151 (1901).
[259] T. P. Hilditch and J. A. Lovern, *J. Soc. Chem. Ind. (London)* **48**, 359T (1929).
[260] N. R. Ellis and O. G. Hankins, *J. Biol. Chem.* **66**, 101 (1925); N. R. Ellis and H. S. Isbell, *ibid.* **69**, 219, 239 (1926).
[261] J. A. Lovern, *Biochem. J.* **32**, 1214 (1938).

and glycogen[262] They tend to be collected into groups forming adipose tissue which occurs subcutaneously around the genital organs, kidney, and other viscera, in the mesentery, between and around the skeletal muscles, and in the omentum. *Deposits also occur in the eye socket and in the bone* marrow cavity of the long bones in adults. Certain special collections of adipose tissue, such as the interscapular pads, may be concerned with vitamin and glycogen storage and hibernation This fat tends to be browner than normal adipose tissue and is found in the mouse, the rat, the hedgehog and many other animals, it is rudimentary in man Brown fat from the hedgehog is said to cause a lowering of the metabolic rate in rats.[263, 264] The distribution of fat in the various depots of the young adult male rat is 50% subcutaneous, 20% in the genital depots, 11% perirenal, 10% mesenteric, 5% intramuscular, and 3% omental.[265] The vascularity of fat depots was, at one time, thought to be poor, but it has been shown recently that adipose tissue has a blood supply comparable with that of skeletal muscle.[266]

The relative distribution between different fat depots varies in the two sexes *and can be affected by estrogens. The subcutaneous fat* is usually greater in women than in men and certainly more evenly spread Fat deposition in the breast occurs characteristically in girls at puberty, and it can also be induced in the male by the administration of synthetic estrogens Increase or decrease of the fat depots tends to occur in a definite order characteristic of the animal.

(3) *Deposition and Mobilization of Fat.* The adipose tissue cells appear to be able to synthesize fats from carbohydrates, or to remove preformed fat from the blood. From general considerations, the supply of suitable precursors and endocrine factors might be expected to affect the amount of fat synthesized in the fat depots Thus, an excessive carbohydrate intake results in increased fat formation, and changes in appetite undoubtedly play an important part in obesity or emaciation associated with pituitary or hypothalamic lesions. Insulin causes a definite increase of fat synthesis.[267, 268] Although cortisone does not favor fat synthesis,[98] the adrenal glands are alleged to play a part in maintaining the fat depots,[269] but their precise relationship to the fat content of adipose tissue is not yet clear.

The amount of preformed fat deposited in adipose tissue is mainly re-

[262] E Tuerkischer and E Wertheimer, *J Physiol* **110**, 385 (1942)
[263] W Eger, *Klin Wochschr* **17**, 1033 (1938)
[264] C F Wendt, *Z physiol Chem* **249**, 4 (1937)
[265] L L Reed, F Yamafuchi, W E Anderson, and L B Mendel, *J Biol Chem* **87**, 147 (1930)
[266] [illegible]
[267] [illegible] 55 (1950)
[268] [illegible]
[269] F [illegible]

lated to dietary glyceride intake, although the amount of particulate fat in the blood after a standard fat meal may vary in different individuals[270] The rate of removal of blood fat into the depots may also differ In patients with idiopathic hyperlipemia glycerides are removed more slowly than normal and accumulate in the blood[271] Heparin causes a rapid clearance of an exogenous lipemia, but it is not known whether this effect is due to more rapid fat deposition or other causes

It has been shown by studies with labeled materials that fats are being continually mobilized from the depots The half-life of the fat depots in rats has been estimated to be 6 to 8 days[272] The mechanism by which this mobilization occurs and the pathway taken by the fat are unknown An increase of blood glycerides of endogenous origin is commonly taken to indicate enhanced mobilization from the depots This blood fat increase, however, may be due to faulty removal of the fat from the blood with a normal rate of mobilization Actual turnover of fat has not been extensively studied, and even this is complicated by the fact that the depots contain a mixture of synthetic and exogenous fat, the proportions of which may vary It is generally thought, however, that fat mobilization is increased by anterior pituitary hormones[273, 274] More rapid depletion of the depots in starvation is claimed after adrenalectomy[275] in rats, but its significance is not clear Nervous regulation of depots has also been demonstrated, and changes in fat mobilization follow section of the spinal cord[276] Lesions of the ventromedial nucleus of the hypothalamus may cause obesity to develop, and the rate of removal of labeled fat from the depots in these obese animals appears to be slower than normal[277]

The amount of fat in the adipose tissues depends upon the balance of income and expenditure An imbalance of food intake in relation to metabolic requirements—as may occur with overeating, especially in sedentary workers, thyroid insufficiency, starvation, or thyrotoxicosis—must inevitably lead towards obesity or emaciation Appetite may become pathological and no longer related to food requirements This may account for the fat changes observed in animals with hyperphagia or hypophagia following hypothalamic lesions,[278] and many cases with pituitary or hypothalamic

270 A C Frazer and H C Stewart, *J Physiol* 95, 23P (1939)
271 M M Stanley and S J Thannhauser, *J Lab Clin Med* 34, 1634 (1949)
272 R Schoenheimer and D Rittenberg, *J Biol Chem* 121, 249 (1936)
273 D Stetten and J Salcedo, *J Biol Chem* 156, 27 (1945)
274 J Campbell and C C Lucas, *Biochem J* 48, 241 (1951)
275 H C Stoerk and C C Porter, *Proc Soc Exptl Biol Med* 74, 65 (1950)
276 E Wertheimer, *Arch ges Physiol* (Pflüger's) 213, 262 (1926)
277 H Mankin, J A F Stevenson, J R Brobeck, C N H Long, and D Stetten, *Endocrinology* 47, 443 (1950)
278 B K Anand and J R Brobeck, *Proc Soc Exptl Med Biol* 77, 323 (1951)

abnormalities There is no doubt, however, that genetic factors may also play a part, since yellow mice become obese on a synthetic diet, but their non-yellow litter mates do not [279]

d. Hyperlipemic Blood Plasma. (1) *Lipid Components and Structural Relationships* The essential difference between alipemic and hyperlipemic blood is an increase in triglyceride fat and the development of a milky appearance There is usually a concomitant, but smaller, increase in both phospholipid and cholesterol. Milkiness may not develop immediately as the glyceride increases, especially if there is an abnormally high phospholipid level [171, 280, 281] Except under these special conditions, however, hyperlipemia may be regarded as the addition of particulate triglyceride to fasting blood plasma These fine particles have an average diameter of about 0 5 μ, but some may be as small as 350 Å, which is the same size as the α-lipoprotein. The particles are easily visible under dark-ground illumination and show active Brownian movement and refractile properties dependent upon size The fat particles are negatively charged, give flocculation characteristics similar to globulin, and migrate with this fraction on electrophoresis, and they remain discrete when packed together by centrifugation The chylomicrons are thought to have a stabilizing film of globulin on the surface [282, 283] Lecithin is also important for chylomicron stability, since the addition of D-lecithinase to hyperlipemic serum causes rapid flocculation and creaming of the fat particles This effect occurs more rapidly than the Nagler reaction [169, 170, 284]

(2) *Exogenous Hyperlipemia* After a fat-containing meal, human subjects normally show an increase of about 300 to 500 mg per 100 ml of glycerides in the blood The lipid changes during alimentary hyperlipemia may be conveniently studied by the chylomicrograph technique, in which the particles per standard dark-ground field[285, 286] are counted For many reasons, however, this is not a satisfactory method for the quantitative assessment of fat absorption [287] With moderate fat intake the maximum hyperlipemia occurs in 2½ to 3 hr. and the blood fat returns to the fasting level by the fifth hour. With larger quantities of fat, the hyperlipemia may

[279] P F Fenton and C J Carr, *J Nutrition* **45**, 225 (1951)
[280] W R Bloor, *J Biol Chem* **49**, 201 (1921)
[281] F M Allen, *J Metabolic Research* **4**, 613 (1923)
[282] S D Ludlum, A E Taft, and R L Nugent, *Colloid Symposium Annual* **7**, 233 (1929)
[283] J J Elkes, A C Frazer, and H C Stewart, *J Physiol* **95**, 68 (1939)
[284] J J. Elkes and A. C Frazer, *J Physiol* **102**, 24P (1943)
[285] S Gage and P G Fish, *Am J Anat* **34**, 1 (1924)
[286] ... and H C Stewart, *J Physiol* 95, 21P (1939)
[287] ... d M D Thompson,

be more prolonged, which is partly due to delayed gastric emptying, resulting from high fat intake The amplitude of the hyperlipemia varies from one individual to another but remains reasonably constant in the same person, except for a tendency to increase in amplitude as age advances [288, 289] The postprandial hyperlipemia in diabetic subjects is said to be greater than normal and to last longer.[290] Alimentary hyperlipemia varies in different animals. It is easily demonstrated after ingestion of fat in man, dogs, cats, and rats It does not occur after fat feeding in guinea pigs and is variable in rabbits.

Hyperlipemia clearly does not depend only upon the rate of intake of glycerides into the blood but also upon the rate of their removal, the balance between these two rates deciding the amplitude and duration. In cases of idiopathic hyperlipemia, in which the rate of removal of absorbed glycerides from the blood is delayed,[271] the blood glycerides accumulate and may exceed 10 g per 100 ml [291, 292] if fat is eaten, but they fall to normal levels on a low-fat diet Heparin can completely prevent the normal alimentary hyperlipemia, and protamine, which is an antiheparin, has precisely the reverse effect [166, 167, 293] Whether these or related compounds are in any way concerned in the normal mechanism of fat deposition is not yet established.

Fine fat emulsions can be injected intravenously [294] If the fat particles are soap stabilized,[295] flocculation occurs when they are mixed with plasma If phospholipid-stabilized emulsions are used, they can be injected and remain discrete in the plasma [296] A number of artificial emulsions have been studied in animals and man, and, although the particles may remain discrete, they do not necessarily behave in the same way as normal chylomicrons Plasma-protein stabilized or natural chylomicron emulsions tend to disappear quite quickly from the blood almost without trace Certain types of artificial emulsions, however, are taken up into macrophages, especially the Kupffer cells in the liver Whether these differences have any important biological significance remains to be seen [297, 298]

[288] A C Frazer and H C Stewart, *J Physiol* 90, 18 (1937)
[289] G H Becker, J Meyer, and H Necheles, *Gastroenterology* 14, 80 (1950)
[290] E F Hirsch and L Carbonaro, *J Lab clin Med* 36, 835 (1950)
[291] L E Holt, Jr , F X Aylward, and H G Timbres, *Bull Johns Hopkins Hosp* 64, 279 (1939)
[292] R D Lawrence, *Lancet* 1, 724, 733 (1946)
[293] W D Brown, *Proc 18th Intern Physiol Congr* , 1950, p 130
[294] A C Frazer and V G Walsh, *J Pharmacol Exptl Therap* 67, 476 (1939)
[295] J J Elkes A C Frazer, J H Schulman, and H C. Stewart, *Proc. Roy Soc (London)* B184, 102 (1945)
[296] A C Frazer, *Discussions Faraday Soc* 6, 81 (1949)
[297] R G Murray and S Freeman, *J Lab Clin Med* 38, 56 (1951)
[298] J J Elkes, *Brit J Nutrition* 3, 367 (1949)

(3) *Endogenous Hyperlipemia.* An increase of glycerides in the blood may occur in starvation, when clearly the fat must be endogenous The occurrence of hyperlipemia in starvation is rather irregular. In normal people deprived of food, hyperlipemia starts after about 36 hr., but the increase of fat may be transient, lasting an hour or so. This transient hyperlipemia may recur at regular intervals [299] In prolonged starvation, the depots become exhausted and consequently hyperlipemia may cease. Whether increased mobilization from the depots occurs in starvation is not proved Fat passes regularly into the blood from the depots under normal circumstances, and there is evidence of considerable dilution of fat in the chyle during absorption It is possible that in starvation the endogenous changes are revealed, owing to the absence of the normal alimentary hyperlipemia

Marked hyperlipemia may occur in uncontrolled diabetes. The fat is derived from the depots, but the exact reason for its accumulation is still not clear A gross disturbance of fat metabolism is demonstrable in this condition, and the utilization of fat is restricted The situation is rapidly improved by adequate insulin therapy.[300, 301] In some animals, especially hens, administration of large doses of estrogens causes a marked hyperlipemia,[302, 303] but this does not occur in man On the other hand, hyperlipemia may occur in pregnancy and during lactation.[304, 305, 306]

Experimental anemia, due to bleeding, plasmapheresis, phenylhydrazine, or trypanosome infection, may cause a marked hyperlipemia in several different species [307-310] Similar observations have been made in man after hemorrhage and in cases of severe anemia [311, 312] Lipid changes apparently occur when either the plasma proteins or the oxygen-carrying power of the blood is reduced. The effect of decreased plasma proteins on fat mobilization or deposition may account for the hyperlipemia observed

[299] A C Frazer, *J Roy Soc Arts* **96**, 582 (1948)
[300] I M Rabinowitch and E S Mills, *J Metabolic Research* **7**, 87 (1925)
[301] A C Curtis, J M Sheldon, and H C Eckstein, *Am J Med Sci* **186**, 548 (1933)
[302] C Entenman, F W Lorenz, and I L Chaikoff, *J Biol Chem* **122**, 619 (1938), *ibid* **134**, 495 (1940)
[303] O Riddle, *Endocrinology* **31**, 31 (1942)
[304] E Herrman and J Neuman, *Biochem Z* **43**, 47 (1912)
[305] E M Boyd, *J Clin Invest* **13**, 347 (1934)
[306] P J Schaible, *J Biol Chem* **95**, 79 (1932)
[307] T R Boggs and R S Morris, *J Exptl Med* **11**, 553 (1909)
[308] H. Schwarz and H H Lichtenberg, *J Biol Chem* **121**, 315 (1937)
[309] E N Chamberlain and R L Corlett, *Brit J Exptl Path* **13**, 299 (1932)
[310] H Dublin, *J Biol Chem* **33**, 377 (1918)
[311] W R Bloor and D J MacPherson, *J Biol Chem* **31**, 79 (1917)
[312] J. Feigle, *Biochem Z* **115**, 63 (1921)

in lipid nephrosis[313, 314] Volatile anesthetics, especially ether, may give rise to hyperlipemia[315]

e. Liver Fat. (1) *Lipid Components* Normal mammalian liver contains approximately 4% total lipids, half to three-quarters of which is phospholipid, while the remainder consists of glycerides and cholesterol[316, 317] The phospholipids contain a high proportion of stearic acid, which is comparable with that found in the corresponding depot fat, and they also contain more highly unsaturated C_{18}, C_{20}, and C_{22} acids, especially arachidonic acid The phospholipids are essentially lecithins, although sphingosine-containing phospholipids are also found The glycerides of sheep, ox, and pig liver fat contain about 10% of hexadecanoic acid and a further 10% of unsaturated C_{20} and C_{22} acids, with three to four double bonds Palmitic acid forms 20 to 30%—comparable to the corresponding depot fat[318]

Elasmo-branch fish liver oils are of three types, with increasing amounts of unsaponifiable matter in each Some fish, such as the skate, have less than 1% of unsaponifiable matter, mainly sterols, but the gray dogfish and ratfish may have 20 to 30% of unsaponifiable matter, mainly glyceryl ethers, and in the shark family 50 to 80% of the hydrocarbon squalene may be present These differences have been attributed to a progressive tendency to hydrogenation of fats in these groups of fish[62] In the teleostid fish,[319] the liver acts as the major store of fat in many cases, but in others there may be little lipid[320] In those with rich oil stores, the fat mainly consists of glycerides containing, in marine fish, 10 to 15% of palmitic, 12 to 18% of hexadecanoic, and 25 to 30% of unsaturated C_{18} acids, 25 to 30% of unsaturated C_{20}, and 10 to 15% of unsaturated C_{22} The fresh-water fish show the usual increase of the shorter-chain C_{16} and C_{18} acids, with a decrease of C_{20} and C_{22}[251]

The normal liver shows no visible fat in the cells, but under certain circumstances fat appears to be released in some way and becomes stainable This process of fat phanerosis has been attributed to breakdown of intracellular lipoprotein structure[321] Although such gross destructive changes must occur in the final stages of cell necrosis, it seems improbable that they would be observed so early within the cell In view of the con-

[313] A Hiller, G C Linder, C Lundsgaard, and D D van Slyke, *J Exptl. Med* 39, 931 (1924)
[314] E H Fishberg, *J Biol Chem* 81, 205 (1929)
[315] W R Bloor, *J Biol Chem* 19, 1 (1914).
[316] W R Bloor, *J Biol Chem* 80, 443 (1928)
[317] E R Theis, *J Biol Chem* 76, 107 (1928)
[318] T P Hilditch and F B Shorland, *Biochem J.* 31, 1499 (1937)
[319] E André and A Bloch, *Bull soc chim France* 2, 789 (1935)
[320] N Evers and W Smith, *Pharm J* 129, 231 (1932)
[321] R Virchow, Die Cellularpathologie, Berlin, 1859

siderable turnover of lipids within the liver cell under normal conditions and the dependence of this metabolic activity upon complex enzyme systems, it seems likely that the early accumulation of fatty materials within the cell would be an expression of altered balance in the intracellular anabolic and catabolic reactions involving lipids. Accumulation of lipid has been described in yeast cells subjected to nutritional restrictions, which can be attributed to a similar metabolic imbalance.[322]

(2) *Income and Expenditure of Individual Lipids.* The compilation of a balance sheet of the lipids in the liver is difficult because of the complex interrelationships between them and the incomplete information available on many aspects of the problem. In general terms the income of any lipid must be of two types—exogenous and endogenous—and the expenditure similarly can occur in two ways—metabolic change or removal. Transport into the blood alone is not an effective method of expenditure, unless the extrahepatic tissues are making use of the material. Most of the fatty acids come to the liver as glycerides, except for some of the shorter-chain acids, which may be transported in unknown form in the portal blood.[246, 247] The liver esterases are relatively ineffective against long-chain triglycerides,[323, 324] and this essential step in fat metabolism in the liver has been largely ignored for many years Fatty acids are readily broken down to 2-carbon fragments,[60] and, as already described, these may enter the tricarboxylic cycle,[68, 69] or form cholesterol[70] or acetoacetate,[71, 72] or be resynthesized back into long-chain fatty acids.[27] Under normal circumstances the amount of ketones formed is probably small, but, if the condensation of the 2-carbon fragments with oxaloacetate is prevented, they may condense to form large quantities of acetoacetic acid.[79, 80] As this cannot be utilized in the liver, it passes out into the blood and is used by the extrahepatic tissues. This is the situation in diabetes and in starvation In the former there is also a blockage of fat resynthesis,[44, 45] while cholesterol formation may be increased. The administration of insulin corrects these metabolic faults. Similar lipid changes occur in Van Gierke's (glycogen storage) disease, but in this case associated with hypoglycemia, and not relieved by insulin[112]

Phospholipid turnover is greater in the liver than in any other organ, and it appears to be the main source of plasma phospholipid in most animals and birds. If the liver is removed after labeling the plasma phospholipids, the rate of their disappearance is extremely slow. Thus the liver is not only the main site of formation but also of removal of plasma phospholipids[325] The significance of this lecithin utilization in the liver in physio-

322 [illegible] *J.* 32, 1571 (1938)

323 [illegible]

324 [illegible]

325 [illegible] 1946)

logical terms is unknown. Phospholipids in the liver commonly contain arachidonic acid, which only slowly declines on a diet free from unsaturated acids. This type of lecithin may be present as a lipoprotein complex in mitochondria and be thus protected from oxidation.[326] Whether this phospholipid is concerned in the normal turnover, or whether other phospholipid molecules or different parts of the lecithin molecule turn over at different rates is not yet clear.

Cholesterol turnover appears to be somewhat slower,[97, 109, 110] but again the liver is the main organ concerned. The income is largely derived from synthesis but also from dietary cholesterol.[70] Expenditure is by transport into the blood, with slow utilization by the tissues, by destruction, and by excretion into the bile.[95, 99, 100] Cholesterol may increase in the liver in diabetes,[44] and in biotin fatty livers, which respond to inositol.[327, 328]

(3) *Accumulation of Lipids in the Liver.* Under certain nutritional circumstances lipids are found to accumulate in the liver in the absence of any gross change in blood lipid levels. This accumulation of lipid must clearly be due either to excessive synthesis of lipids in excess of removal, or to faulty catabolism or transport. The most important form of liver fat accumulation is that observed in animals fed an alipotropic diet. The essential deficiency in this diet has been shown to be lack of choline.[329, 330] Similar effects are also produced by diets deficient in transmethylating agents[331, 332] such as methionine, which is probably related to the same mechanism.[333, 334] Cystine has an antagonistic action to methionine.[335] If animals are fed a diet lacking in choline, glyceride accumulates in the liver and may reach more than ten times the normal level. The fat is intracellular, and gradually the droplets coalesce until the whole cell is filled with fat. As the process continues the cells rupture and cysts form, filled with fat, to which as many as 60 to 80 cells may contribute. If choline is restored while the fat is still intracellular, the fat rapidly disappears, but the cysts change more slowly.

[326] E. P. Kennedy and A. L. Lehninger, *Federation Proc.* **8**, 213 (1949).
[327] G. Gavin and E. W. McHenry, *J. Biol. Chem.* **132**, 41, (1940), *ibid.* **139**, 485 (1941), **141**, 619 (1941).
[328] C. H. Best, C. C. Lucas, J. M. Paterson, and J. H. Ridout, *Biochem. J.* **40**, 368 (1946).
[329] C. H. Best, J. M. Hershey, and M. E. Hunteman, *J. Physiol.* **75**, 56, 405 (1932).
[330] C. H. Best and C. C. Lucas, Vitamins and Hormones **1**, 1 (1943).
[331] V. du Vigneaud, J. P. Chandler, M. Cohn, and G. B. Brown, *J. Biol. Chem.* **134**, 787 (1940).
[332] V. du Vigneaud, M. Cohn, J. P. Chandler, J. R. Schenk, and S. Simmonds, *J. Biol. Chem.* **140**, 625 (1941).
[333] H. J. Channon, M. C. Manifold, and A. P. Platt, *Biochem. J.* **32**, 969 (1938).
[334] H. F. Tucker and H. C. Eckstein, *J. Biol. Chem.* **121**, 479 (1937).
[335] C. R. Treadwell, M. Groothuis, and H. C. Eckstein, *J. Biol. Chem.* **142**, 653 (1942).

It is suggested that the cysts may rupture into blood vessels and give rise to multiple fat emboli.[336] If choline is withheld from the diet, the turnover of choline-containing phospholipid is reduced; if choline is added, the turnover of these phospholipids is stimulated at the expense of the non-choline-containing group.[133] Labeled choline has been traced into the phospholipid molecules in the liver.[125] There seems little doubt, therefore, that dietary choline is concerned with the formation of choline-containing phospholipids in the liver, especially lecithin. The fat accumulates as glyceride *within the cell, so that there is no interference with the passage of glyceride* through the cell wall from without inwards. As already stated, transport of lecithin from the cell is not an effective form of expenditure, since it is used so slowly by the extrahepatic tissues.[125] It would seem, therefore, that lecithin must play an important part in the early stages of the metabolism of glyceride in the liver. The fat may be derived from the depots or by synthesis from carbohydrate—that the latter may be concerned is suggested by the observation that dietary vitamin B levels affect fat accumulation in choline deficiency.[337] Fat accumulation occurs with ingestion of a high fat diet,[338] but it is preventable with choline, since this presumably enables expenditure to keep pace with increased income. The type of fat fed also affects fatty liver formation, butter being most effective.[339] The vitamin B group appears to be closely associated with the development of fatty livers, but the mechanism of action in each case is still not clear.[340-343] It is probable that fatty livers associated with chronic poisoning with alcohol are largely attributable to nutritional deficiencies. A number of extracts have been prepared from the pancreas which appear to affect fat accumulation in the liver, most of the action of these substances has been satisfactorily accounted for on the basis of choline, protein, or proteolytic enzyme content.[344-350] Interference with the normal oxidative mechanisms in the liver

336 W. S. Hartroft, Ciba Foundation Symposium on Liver Disease. Churchill, London, 1951, pp. 90, 97

337 E. W. McHenry, *J. Physiol.* **86**, 27P (1936)

338 E. Flock, J. L. Bollman, H. R. Hester, and F. C. Mann, *J. Biol. Chem.* **121**, 117 (1937)

339 H. J. Channon and H. Wilkinson, *Biochem. J.* **30**, 1033 (1936)

340 W. H. Sebrell, Jr. and R. H. Onstott, *Pub. Health Rept.* **53**, 83 (1938)

341 P. György, *Am. J. Clin. Path.* **14**, 67 (1944).

342 M. M. Burns and J. M. McKibbin, *J. Nutrition* **44**, 487 (1951)

343 B. Kelley, J. R. Totter, and P. L. Day, *J. Biol. Chem.* **187**, 529 (1951)

344 L. R. Dragstedt, V. Prohaska, and H. P. Harms, *Am. J. Physiol.* **117**, 175 (1936)

345 F. X. Aylward and L. E. Holt, *J. Biol. Chem.* **121**, 61 (1937)

346 [illegible] 410 (1937)

347 [illegible]

348 [illegible]

349 [illegible] (1951)

350 M. L. Haanes and P. György, *Am. J. Physiol.* **166**, 441 (1951).

cells may result in fat accumulation, or, if sufficiently severe, in gross disorganization and necrosis. As already described, inability to deal with the 2-carbon fragments from fatty acid degradation, either by condensation with oxaloacetate and entry into the tricarboxylic acid cycle, or by resynthesis to long-chain fats, results in excessive formation of cholesterol and acetoacetic acid in the diabetic liver. A number of poisonous substances interfere with the oxidative enzyme systems of the liver, with consequent disturbance of the normal metabolic activities which may lead to lipid accumulation. In all cases in which lipids accumulate in the liver cells for any length of time, diffuse hepatic fibrosis is likely to ensue.[111]

3. Secretion and Excretion of Lipids

a. Secretion of Lipids. (1) *Milk.* Milk, the natural food of the infant animal, contains glycerides, phospholipids, and cholesterol. The glycerides are present in amounts of 3.5% up to 15% or more in different animals. A high fat content is found in smaller animals, running more or less parallel with the protein content, which seems to vary in relation to the rate at which the animal doubles its birth weight. This is achieved in the rat in 4 to 5 days; the human infant takes 180 days. High fat content is also found in animals living in a cold environment, such as the reindeer and the whale. The phospholipid content is usually low, being about 0.05% in cow's milk, and the cholesterol content is less, but these lipids are abundant in colostrum.[112] The fatty acids in the milk of cows, goats, sheep, and other herbivores include a significant proportion of the shorter-chain fatty acids which are absent from human milk.[79, 113]

The origin of milk fat has been the subject of controversy for some years. There seems no doubt that milk fat can be influenced by food fat and[114] therefore must, at least in part, be derived from preformed fat. However, dextrose feeding causes a marked increase in milk fat formation.[115] The origin of milk fat may differ in the cat, the dog, and the rat from the goat and the cow, being largely derived from blood fat in the former but synthesized from carbohydrate in the mammary gland in the latter.[24] Recent work using isotope-labeled materials shows that fatty acids and cholesterol can be synthesized from 2-carbon fragments in the mammary gland. The fatty acids are built up by the addition of 2-carbon fragments and all the saturated fatty acids, up to and including palmitic acid, have been

[111] H. P. Himsworth, The Liver and Its Diseases. Blackwell, Oxford, 1947.

[112] B. Nims, I. G. Macy, M. Brown and H. A. Hunscher, *Am. J. Dis. Child* 43, 828, 1932.

[113] A. W. Bosworth and J. B. Brown, *J. Biol. Chem.* 103, 115 (1933).

[114] T. P. Hilditch and H. E. Longenecker, *J. Biol. Chem.* 122, 497 (1938).

[115] F. H. Smith, C. A. Wells and P. V. Ewing, *Georgia Expt. Sta. Bull.* 122 (1915).

[116] M. Bürger and W. Puckert, *Z. physiol. Chem.* 196, 193 (1931).

It is suggested that the cysts may rupture into blood vessels and give rise to multiple fat emboli.[336] If choline is withheld from the diet, the turnover of choline-containing phospholipid is reduced; if choline is added, the turnover of these phospholipids is stimulated at the expense of the non-choline-containing group.[138] Labeled choline has been traced into the phospholipid molecules in the liver.[125] There seems little doubt, therefore, that dietary choline is concerned with the formation of choline-containing phospholipids in the liver, especially lecithin. The fat accumulates as glyceride *within the cell, so that there is no interference with the passage of glyceride* through the cell wall from without inwards. As already stated, transport of lecithin from the cell is not an effective form of expenditure, since it is used so slowly by the extrahepatic tissues.[125] It would seem, therefore, that lecithin must play an important part in the early stages of the metabolism of glyceride in the liver. The fat may be derived from the depots or by synthesis from carbohydrate—that the latter may be concerned is suggested by the observation that dietary vitamin B levels affect fat accumulation in choline deficiency.[337] Fat accumulation occurs with ingestion of a high fat diet,[338] but it is preventable with choline, since this presumably enables expenditure to keep pace with increased income. The type of fat fed also affects fatty liver formation, butter being most effective.[339] The vitamin B group appears to be closely associated with the development of fatty livers, but the mechanism of action in each case is still not clear.[340-343] It is probable that fatty livers associated with chronic poisoning with alcohol are largely attributable to nutritional deficiencies. A number of extracts have been prepared from the pancreas which appear to affect fat accumulation in the liver; most of the action of these substances has been satisfactorily accounted for on the basis of choline, protein, or proteolytic enzyme content.[344-350] Interference with the normal oxidative mechanisms in the liver

[336] W. S. Hartroft, Ciba Foundation Symposium on Liver Disease. Churchill, London, 1951, pp. 90, 97

[337] E. W. McHenry, *J. Physiol.* **86**, 27P (1936)

[338] E. Flock, J. L. Bollman, H. R. Hester, and F. C. Mann, *J. Biol. Chem.* **121**, 117 (1937)

[339] H. J. Channon and H. Wilkinson, *Biochem. J.* **30**, 1033 (1936)

[340] W. H. Sebrell, Jr. and R. H. Onstott, *Pub. Health Rept.* **53**, 83 (1938)

[341] P. György, *Am. J. Clin. Path.* **14**, 67 (1944)

[342] M. M. Burns and J. M. McKibbin, *J. Nutrition* **44**, 487 (1951)

[343] B. Kelley, J. R. Totter, and P. L. Day, *J. Biol. Chem.* **187**, 529 (1951)

[344] L. R. Dragstedt, V. Prohaska, and H. P. Harms, *Am. J. Physiol.* **117**, 175 (1936)

[345] F. X. Aylward and L. E. Holt, *J. Biol. Chem.* **121**, 61 (1937)

[346] E. M. Mackay and R. H. Barnes, *Proc. Soc. Exptl. Biol. Med.* **38**, 410 (1937)

[347] C. H. Best and J. H. Ridout, *Am. J. Physiol.* **122**, 67 (1938)

[348] I. L. Chaikoff and C. Entenman, *Advances in Enzymol.* **8**, 171 (1948)

[349] D. K. Bosshardt, L. S. Ciereskko and R. H. Barnes, *Am. J. Physiol.* **166**, 433 (1951)

[350] M. L. Haanes and P. György, *Am. J. Physiol.* **166**, 441 (1951

developing egg occurs between the fifteenth and seventeenth days of incubation[368]

(3) *Sebum and Skin Fats* An appreciable amount of cholesterol and traces of fatty acids and the hydrocarbon, squalene, are secreted daily by the skin[369] This lipid material is important for the normal health of the skin, and the fatty acids have been shown to possess bacteriocidal properties[370]

b. Excretion of Lipids. (1) *Bile* The bile contains about 0 05% of cholesterol in fistula samples and 0.5 to 1% in bladder bile. Cholesterol feeding appears to increase the amount in the bile[371] Some of the cholesterol is absorbed from the intestine, and the remainder is converted to coprosterol by the intestinal bacteria.[98, 99, 100] Phospholipid, fatty acids, and soaps are also present in bile The lipids of bile are decreased in choline deficiency[372]

(2) *Fecal Lipids* On a diet containing 50 g of fat a normal human subject does not pass more than 5 g of fatty acids in the stools in each 24 hr. The assessment of fecal fat for balance purposes is best carried out for each 24-hr. period and assessed as a 3-day sliding mean to correct for sampling errors Estimation of total fatty acid is usually sufficient, since information on percentage hydrolysis or soap content is of little value The fatty acids are mainly saturated long-chain fatty acids and a small quantity of volatile fatty acids not exceeding 1 g per 24 hr[287, 373, 374] These volatile fatty acids come from carbohydrate, while the long-chain fatty acids may be derived from four possible sources—residual dietary fat, bacteria, epithelial debris, and excreted fat The fecal fat increases slightly with changed dietary fat levels, which indicates that unabsorbed fat makes some contribution[375] Bacteria certainly contain lipids in their bodies, fatty acid forming up to 5% of the dry weight of enteric organisms, and some of the intestinal bacteria and yeasts can synthesize fats[376, 377, 378] Epithelial debris is more likely to account for a significant proportion of the unsaponifiable residue

368 O E Kugler, *Am J Physiol* **115**, 287 (1936)
369 R M B McKenna, V R Wheatley, and A Wormall, *J Investigative Dermatol* **15**, 33 (1950)
370 C R Rickets, J R Squire, and E Topley, *Clin Sci* **10**, 89 (1951)
371 P D McMaster, *J Exptl Med* **40**, 25 (1924)
372 A R Colwell, Jr, *Am J Physiol* **164**, 274 (1951)
373 W T Cooke, J J Elkes, A C Frazer, J Parkes, A L Peeney, H G Sammons, and G Thomas, *Quart J Med* **39**, 141 (1946)
374 J H van de Kamer, H Ten B Huinink, and H A Weyers, *J Biol Chem* **177**, 347 (1949)
375 E E Wollaeger, W O Lundberg, J R Chipault, and H L Mason, *Federation Proc* **10**, 271 (1951)
376 H C. Eckstein and M H Soule, *J Biol Chem* **91**, 395 (1931)
377 C H Williams, W R Bloor, and L A Sandholzer, *J Bact* **37**, 301 (1939)
378 I Smedley-Maclean and D Hoffert, *Biochem J* **20**, 343 (1926)

formed[357, 358] It seems likely that synthesis from short-chain fragments is important in herbivorous animals for milk fat formation, but in omnivores and carnivores a significant proportion of the milk fats may be derived from the body lipids.

(2) *Egg Yolk.* The hen's egg contains about 10% of lipid. The yolk is 35% lipid, of which 10 to 15% is phospholipid and the remainder glyceride and cholesterol. The constituent fatty acids of the phospholipids appear to be similar to those in the liver of land animals, and the glycerides resemble the fat depots of the hen The proportion of lecithins to cephalins is 3:1. Much of the lipid in egg yolk is closely associated with protein, forming lipovitellin. Autoxidation of the original lipoprotein or its separated components is similar, so that the association with protein does not appear to protect the phospholipid in this case.[359]

The diet of the hen has a profound effect upon the egg lipids,[355, 360] especially with regard to unsaturated fats.[361] Feeding a fat-free diet markedly affects the iodine value of the egg fats—both glycerides and phospholipids Hens on a fat-free diet lay eggs deficient in polyunsaturated fatty acids, but egg production and hatchability remain normal On replacing unsaturated fatty acids individually in the diet, it is found that dienoic acids give rise to pentaenoic and, possibly, tetraenoic acids, while trienoic acids give rise to all polyethenoid acids with 2 to 6 double bonds Ingested conjugated dienoic and trienoic acids are deposited in the egg and converted to other unsaturated acids[362, 363]

Lecithin from the hen egg yolk contains palmitic, stearic, oleic, linoleic, and clupanodonic acids,[364] and the ratio of total phospholipid to lecithin content is 1.45 in the hen, 1 35 in the duck, 1 2 in the peacock, and 1 8 in the quail The same ratio was found to be 1 0 in shark eggs, 1.1 in salmon, carp, and prawn, 1 6 in cod, and 2 4 in herring[365] Cholesterol content of the yolks of many different types of bird eggs remains fairly constant at about 1 to 2%[366] Snake eggs have a similar composition to the hen's egg, with rather less phospholipid.[367] Maximum phospholipid metabolism in the

357 G Popják, T H French, and S J Folley, *Biochem J* **48**, 411 (1951)

358 G Popják, T H French, G. D Hunter, and A J Martin, *Biochem J* **48**, 612 (1951)

359 [illegible] J C Hawke, *Biochem J* **50**, 67 (1951)

360 [illegible]

361 [illegible]

362 [illegible]

363 [illegible]

364 [illegible] *Biol Chem* **126**, 255 (1938)

365 [illegible] *J Agr Chem Soc Japan* **13**, 200 (1937)

366 [illegible]

367 [illegible]

1. Demand for Lipids by the Tissues

There is a threefold demand for lipids—for growth and tissue replacement, for energy, and for secretions.

a. Growth and Tissue Replacement. Lipids play an important part in the basic structure of protoplasm—the acetal phosphatides,[175] proteolipids,[176] and strandin[177] are examples of complex lipid molecules that seem to be present in ground substance. The cell cytoplasm in animal tissues contains particulate structures, such as microsomes, mitochondria, and Golgi elements, that play a vital part in the life of the cell and contain significant quantities of lipids, mainly phospholipids.[180, 183, 188] The fatty acids in these phospholipids include a high proportion of polyethenoid acids, especially arachidonic acid.[181] These highly unsaturated lecithins may be protected from oxidation by association with protein,[326] or antioxidants, since free unsaturated fatty acids are said to be readily broken down in the tissues. The cells are commonly enclosed in a cell membrane consisting of lipid and protein molecules. The cell membrane of the erythrocyte has been extensively studied, and the main lipids present are cephalins and sphingomyelins.[191] If the lipids are removed or displaced, marked changes in permeability of the membrane occur.[201, 202] Lipids are also concerned with the structure of neurons, lecithin being an important constituent of axoplasm, but cephalins, sphingomyelins, and cerebrosides predominating in myelin.[204] The structural integrity of the myelin sheath is essential to the normal activity of the myelinated neurone. If the neurone is damaged, the myelin structure breaks down, but it can be regenerated under suitable conditions in peripheral nerve, but not in the central nervous system.[208, 209] The lipids of myelin contain little polyethenoid fatty acid, so that myelination, in contrast to general growth of the nervous system, does not set up a great demand for unsaturated fatty acids. In fasting blood plasma, at least 75% of the lipids are present as lipoproteins.[160] These lipids are essentially phospholipid, especially lecithin, and both free and esterified cholesterol. Although these lipids may be concerned in the accommodation of further lipid in the blood plasma, they are not themselves readily utilized in the tissues and may be properly regarded as structural lipids.

In animal cells there are two forms of turnover to be considered—molecular and cellular. It is probable that individual molecules in any cell can be replaced by new molecules, and this process of replacement is going on continually without necessarily involving any change in the normal life and activity of the cell. If this process were completely efficient, all cells would be potentially immortal. This is not the case with red or white blood corpuscles, stratified epithelium, or intestinal cells. Most cells, except the neurons of the central nervous system, are probably being continually replaced. There is, thus, a general turnover of cells, as well as molecules, the rate varying considerably from one tissue to another.

and might contain small amounts of fatty acid Fat excretion is still an unknown factor.

Under certain circumstances the amount of fat in the stools may be greatly increased, the commonest conditions being the sprue group, surgical conditions of the intestine such as gastrocolic fistula, diabetes, pancreatitis, regional ileitis, and Whipple's disease. The reason for the increase in fecal fat in many of these cases is still obscure. Most of the sprue and pancreatic cases have a definite fat absorption defect.[379] An increase of non-dietary fat in the feces has also been described in some cases of celiac disease[380] and after pancreatectomy in dogs,[381] but this is not necessarily due to increased fat excretion

Unsaponifiable matter accounts for nearly half of the ether-soluble material in the stools. It seems to have little relationship to diet Although it can be shown that neither bile, diet, nor bacteria could account independently for the unsaponifiable matter in the feces,[382, 383] each makes an important contribution, and the exclusion of one of them may cause a quantitative alteration in the others.

(3) *Urine* A few milligrams of fat are commonly found in the urine, and this may be increased in renal disease[384] In chyluria large amounts of fat may be passed, depending upon the fat content of the food[385] In the cat family, deposition of fat in the kidney occurs in relation to the sexual cycle, and the tiger may pass quite considerable quantities of fat.[386, 387]

III. Nutritional Implications

Consideration of the functions of lipids in the body and the changes they may undergo indicates the main nutritional implications of lipid metabolism—*the demand for lipids by the tissues, the supply of lipids to meet this demand and the possible deleterious effects which dietary lipids may cause*

379 A C Frazer, Parsons' Modern Trends in Paediatrics, Butterworth, London, 1951, p 528

380 H A Weyers and J H van de Kamer, Centraal Instituut voor Voedingsonderzoek T N O, *Publication 113, Utrecht, 1950*

381 C Jiménez Díaz, C Marina, and J M Romeo, *Bull. Inst Med Res (Madrid)* **2**, 219 (1949), *ibid* **3**, 165 (1950)

382 W M Sperry, *J Biol Chem* **68**, 357 (1926), *ibid* **71**, 351 (1927), *ibid* **96**, 759 (1932)

383 W M Sperry and R W Angevine, *J Biol Chem* 96, 769 (1932)

384 … *Soc Exptl Biol Med* **17**, 115 (1920),

385 C F Asenjo, J E Colon, and I Hernandez-Morales, *Puerto Rico J Pub Health, Trop Med* **26**, 55 (1950)

386 M C Lobban, *J Physiol* **112**, 8P (1950)

387 T Hewer, L H Matthews, and T Malkin, *Proc Zool Soc London* **118**, 924 (1948)

gram of fat provides nearly twice as many calories as a similar weight of carbohydrate or protein, the advantages of fat storage are apparent. Furthermore, any surplus carbohydrate can also be converted into fat in the adipose tissue cells [262]

c. Lipid Secretions. The tissue cholesterol is the mother substance of many steroid hormones and also[101, 102, 103] vitamin D_3 [104] There must therefore be a continuous demand for cholesterol, since after its modification into the biological active form it probably undergoes further change into an inactive molecule which is either excreted or destroyed The demand for cholesterol increases when the endocrine glands secreting steroid hormones are stimulated[218] or in an active phase [221]

The main lipid-containing exocrine secretions are milk,[352] egg yolk[360, 361] and sebum [369, 370] There is an obvious demand for glycerides, phospholipids, and cholesterol during lactation in mammals and the laying season in many other animals Secretion of skin fats is a small but definite demand, in wool-covered animals the amounts of lipid involved may be quite considerable

2 Supply of Lipids

The necessary demand for lipids can be met in three ways—by biosynthesis, by dietary intake, and by redistribution Substances that cannot be synthesized in the animal body are dietary essentials Lipid biosynthesis itself raises special problems in nutrition, since the presence or absence of certain dietary constituents may profoundly influence these metabolic reactions

a. Biosynthesis. (1) *Energy Lipids* All the fatty acids required for energy production can be synthesized from suitable 2-carbon fragments which can be derived from the degradation of a wide range of organic compounds [30] These can be readily built up into glycerides in adipose tissue cells The amount of carbohydrate and fat in the combustion mixture can thus be adjusted within certain limits, surplus carbohydrate being stored as fat until required Thiamine plays an important part in the mechanism of conversion of carbohydrate to fat, since it is a coenzyme for pyruvic acid decarboxylase [33]

(2) *Structural Lipids and Sterol Hormones* Cholesterol can be synthesized *in situ* for structural purposes or for the elaboration of steroid hormones. A number of different short-chain fragments are suitable for this purpose, and synthesis is stimulated by cortisone [95] Active cholesterol synthesis occurs in skin, ovaries, and adrenals, but not in nervous tissue. It is interesting that integrity of the cell is necessary for both cholesterol and phospholipid synthesis.[92, 95, 131, 132]

Phospholipid formation is more complicated, since a number of important dietary constituents are required as starting materials The fatty acids may

During all periods of active growth involving the formation of new cells there is an absolute demand for lipids by the tissues. This demand must vary with changes in growth rate. The lipids required are long-chain fatty acids, including the polyethenoid series, which are incorporated into various phospholipids, cholesterol, and cerebrosides. From the beginning, cells are being replaced—white blood cells only live a few hours—so that, quite apart from any actual increase in the number of cells, there is a constant demand for lipid replacement which will continue after somatic growth is complete. This demand will be greater if there is accelerated tissue destruction, such as may follow heavy irradiation with X-rays.

b. Energy Production. The main lipids used for energy production are the saturated and monoethenoid fatty acids from acetic up to the C_{18} acids. The polyethenoid and longer-chain saturated fatty acids are not normally used for energy purposes. Fatty acids can be broken down and utilized both in the liver and the extra-hepatic tissues, at least 60% of fat oxidation normally occurring in the liver.[65] Fats are being used continually, since the respiratory quotient of most animals is less than 1. The demand for energy fats increases if there is a shortage of available carbohydrate—this occurs in starvation when the glycogen stores are exhausted and in diabetes due to lack of insulin. It can also be observed in certain animals during hibernation, when the respiratory quotient may be as low as 0.3.[338]

Some controversy has arisen over the mechanism of transport of energy fats to the tissues largely because it was thought that the fat must pass first to the liver for desaturation. The plasma lecithin was considered to be a fat transport mechanism, but its removal from the blood practically ceases if the liver is removed.[325] There is little evidence that cholesterol esters transport energy lipids. Since preliminary desaturation in the liver is not necessary and the tissues can utilize fatty acids and glycerides direct,[62, 63, 64] there is no need for any special transport mechanism, and particulate triglyceride would seem to be the normal form in which the energy fats are redistributed in the body.[339] Some fatty acids may pass direct to the liver from the intestine, but the form in which they are transported is unknown.[246, 247] Acetate and acetoacetate may constitute important forms [illegible] but,

these early stages.

Only a proportion of glycerides are used for immediate metabolic needs, and the remainder is deposited in the fat depots for future use. Since every

[338] M. S. Pembrey and P. A. Gorer, *J. Physiol.* 67, 21P (1929).
[339] A. C. Frazer, *Discussions Faraday Soc.* 6, 81 (1949).

donic acid and other necessary polyethenoid fatty acids Arachidonic acid is an essential constituent of intracellular lecithins Animals placed on a fat-free diet develop skin lesions, growth is stunted, and no arachidonic or more unsaturated fatty acids are found in the tissues [55, 392-395] The signs of fat deficiency can be precipitated in adult animals by X-irradiation,[396] but not by administration of growth hormone [397] The two commonest dietary fatty acids containing this grouping are linoleic and linolenic acid, and these have been shown to relieve the effects of fat deficiency and to give rise to arachidonic acid formation in the tissues [398, 399] The double bonds in these acids may be protected in some way in the body and the tetraenoic acid is presumably formed by condensation of suitable unsaturated fragments The unsaturated fats of fish oils often contain fatty acids with a $-CH\ CH\ CH_2\ CH_2\ CH{\cdot}CH-$ group which are no use for relief of fat deficiency However, cod-liver oil and other fish oils also contain clupadonic acid and other polyethenoid fatty acids which contain both forms of unsaturated groupings and are consequently effective Elaeostearic acid is a trienoic acid with the grouping $-CH.CH\ CH{:}CH\ CH{:}CH-$, and this cannot replace linoleic acid in the diet [398] Arachidonic acid itself will relieve fat deficiency symptoms [400] The *trans* isomers of essential fatty acids do not relieve fat deficiency [401] The dietary essential, pyridoxin, plays an important part in unsaturated fatty acid metabolism [393, 402]

c. Redistribution of Lipids. The demand for lipids by individual tissues can to some extent be met by redistribution Little is known about the reutilization of lipid molecules for structural purposes Clearly many of these substances derived from obsolete cells are broken down to small fragments which enter the general metabolic pool and may be built into new molecules, this is clearly indicated by the rapid and wide distribution of C^{14} after the administration of labeled molecules In the case of the *energy lipids redistribution is continually taking place from the fat stores* to the tissues [272] It seems probable that fat deposition and mobilization are

[392] H F Wiese and A E Hansen, *Texas Repts Biol Med* **9**, 516, 549 (1951)
[393] G Medes, M V Mann, and J B Hunter, *Arch Biochem Biophys* **32**, 70 (1951)
[394] A E Hansen, S G Holmes and H F Wiese, *Texas Repts Biol Med* **9**, 555 (1951)
[395] P S Menon, P G Tulpule, and V N Patwardhan, *Indian J Med Research* **38**, 173 (1950)
[396] A B Decker, D L Fillerup, and J F Mead, *J Nutrition* **41**, 507 (1950)
[397] S M Greenberg, C E Calbert, E E Savage, and H J Deuel, Jr, *J Nutrition* **41**, 473 (1950)
[398] G O Burr, N M Burr, and E S Miller, *J Biol Chem* **97**, 1 (1932)
[399] L C A Nunn and I Smedley-Maclean, *Biochem. J* **32**, 2178 (1938)
[400] R T Holman and T S Taylor, *Arch Biochem* **29**, 295 (1950)
[401] R T Holman, *Proc Soc Exptl Biol Med* **76**, 100 (1951)
[402] H Schneider, H Steenbock and B R Platz, *J Biol Chem* **132**, 539 (1940)

include long-chain unsaturated fatty acids, such as arachidonic acid, which cannot be synthesized from 2-carbon fragments in the animal body Various bases, such as choline, serine, and ethanolamine, and carbohydrates such as inositol and galactose, are also required These various substances, or suitable precursors, must therefore be included in the diet Assuming that all these necessary components are available, structural phospholipids can be synthesized *in situ* in most tissues[128, 131, 134] Plasma phospholipid does not appear to be concerned in transport to the tissues for either energy or structural purposes. If any of these necessary raw materials is absent, the pattern of phospholipids formed may be correspondingly altered. Thus, in choline deficiency, a greater proportion of non-choline-containing phospholipids may be formed in the liver,[135] and if polyethenoid fatty acids are lacking the resultant phospholipids are more saturated.[139]

(3) *Lipid Exocrine Secretion* In milk formation most of the lipids can be synthesized in the mammary gland. This is especially marked in herbivorous animals[157, 158] The fetus and the young animal are also able to synthesize these lipids It is necessary, however, to have the dietary essentials required for the formation of structural phospholipids—especially the unsaturated fatty acids At the peak of human lactation about 30 to 40 g. of triglyceride are secreted per day In egg yolk, lipids can be synthesized, but lack of unsaturated fatty acids in the diet of the hen results in the absence of these essential lipid constituents from the egg[162, 163]

b. Dietary Lipids. (1) *General Lipid Intake.* The dietary intake of lipids varies greatly, both individually and from one species to another Carnivores and omnivores usually consume more fat than herbivorous animals In man an exceptionally high fat intake is common among Eskimos and lumberjacks working under Arctic conditions—it is probably the only practical means of raising the caloric level of the diet sufficiently high for these people The addition of 10% of cottonseed oil to a synthetic diet containing optimal amounts of linoleic acid causes further growth in rats which is not accounted for on the basis of caloric intake alone.[390] Dogs on a fat-deficient diet return to normal only when 16% of the caloric intake is provided by fat, although the specific effects of essential fatty acid deficiency are relieved by 1% of the caloric intake supplied as linoleic acid[391] Fats may therefore have some nutritional value beyond the provision of the essential fatty acids, improvement in palatability of the diet, and carriage of fat-soluble vitamins

(2) *Polyethenoid Fatty Acids* Animal tissues seem to be unable to synthesize the grouping $-CH{:}CH{\cdot}CH_2$ $CH{:}CH-$ which is found in arachi-

[390] H J Deuel, Jr, S M Greenberg, C. E Calbert, E E Savage and T Fukui, *J Nutrition* 40, 341 (1950)

[391] A E Hansen and H F Wiese, *Texas Repts. Biol Med* 9, 491 (1951)

rial is largely exogenous,[407] although arterial tissues can synthesize lipids [408] For many years it has been thought that some relationship exists between blood lipids, especially blood cholesterol, and atheroma [409-413] It is possible to induce atheromatous changes in experimental animals by causing hypercholesterolemia This can be done by feeding excessive amounts of cholesterol and fat[414, 415] by high estrogen dosage in chicks,[416] and by a number of other methods [417] Atherogenesis varies markedly in different species, the rat being particularly resistant The absolute level of cholesterol in the blood is probably not so important as its relationship to other lipids, or the form in which the cholesterol is carried in the blood As already described, an unstable macromolecule of the Sf_{10-20} group may be an important factor.[173] There is evidence to show that trauma of the endothelial lining of the vessels predisposes to lipid deposition [418, 419, 420] Other workers have stressed the importance of fat in atherogenesis [421, 422, 423, 424] Choline and inositol appear to have no influence on atherogenesis in experimental animals [425-428]

(2) *Gall Bladder Wall* The gall bladder wall appears to be able to absorb lipids, and the bile[429] normally contains cholesterol Under certain circumstances some of this cholesterol may be deposited in the gall bladder wall, or it may be formed into crystalline material in the lumen of the gall bladder Nearly 50% of all persons over 20 have demonstrable lipid deposits in the gall bladder wall It is a matter of some interest that gall bladder

[407] M W Biggs and D Kritchevsky, *Circulation* 4, 34 (1951)
[408] S Chernick, P A Srere, and I L Chaikoff, *J Biol Chem* **179**, 113 (1949).
[409] H B Schmidt, *Arch Intern Med* **13**, 121 (1914)
[410] F D Gorham and V C Meyers, *Arch Intern Med* **20**, 599 (1917)
[411] W Denis, *J Biol Chem* **29**, 93 (1917)
[412] M Labbe and J Heitz, *Compt rend soc biol* **87**, 1024 (1922)
[413] G Blix, *Acta Med Scand* **64**, 142, 175, 234 (1926)
[414] W Hueck, *Verhandl deut path Ges* **20**, 18 (1925)
[415] T Leary, *J Am Med Ass* **105**, 475 (1935)
[416] S Lindsay, F W Lorenz, C Entenman, and I L Chaikoff, *Proc Soc Exptl. Biol Med* **62**, 315 (1946)
[417] J B Firstbrook, *Brit Med J* ii, 133 (1951)
[418] G L Duff, *Arch Path* **22**, 161 (1936)
[419] G I DeSuto-Nagy and L L Waters, *Federation Proc* **10**, 353 (1951)
[420] J H McCormick, C H Lippard, and R L Holman, *Federation Proc* **10**, 363 (1951)
[421] J R Moreton, *Science*, **107**, 371 (1948)
[422] W J Zinn and G C Griffith, *Am J Med Sci* **220**, 597 (1950)
[423] H Necheles, *Am J Digestive Diseases* **18**, 229 (1951)
[424] A Keys, *Proc Internat Dietetic Congr*, Amsterdam (1952)
[425] J D Davidson, W Meyer, and F E Kendall, [illegible]
[426] J Sta [illegible]
[427] J B [illegible]
[428] P H [illegible]
[429] S H [illegible]

under both nervous and endocrine control and that these factors help to maintain a proper balance between the demand and supply of energy materials[273, 274]

3. Deleterious Effects of Dietary Lipid

a. Ketogenesis. With a normal balanced diet containing carbohydrates and fats, the combustion mixture is adjusted so that the 2-carbon fragments derived from long-chain fatty acids are almost entirely taken up into the tricarboxylic acid cycle,[65, 69] and only small amounts of acetoacetic acid appear to be formed. As the proportion of fat to carbohydrate is increased, so less of the 2-carbon fragments are effectively dealt with in the tricarboxylic acid cycle[73] and consequently form increasing amounts of acetoacetic acid by condensation.[71, 72] Thus, a high-fat low-carbohydrate diet causes marked ketosis, which may be accompanied by nausea and vomiting

b. Oxidative Rancidity. Unsaturated fatty acids may undergo oxidative rancidity which gives rise to peroxides and many other derivatives and, when it occurs in glycerides, to polymerization[19, 20, 403] These oxidized fats may cause destruction of other oxidizable dietary components In particular, vitamin A and vitamin E deficiency, due to the presence of rancid fats, has been described[404, 405] These rancid fats have other toxic properties which are not related to destruction of these vitamins and which appear to be retained when the peroxide level has passed its peak.[406] The exact nature of this toxic effect is not known, but it may be related to polymer formation[403] It is of the greatest importance that the dangers of oxidative rancidity to the nutritive value of the diet should be borne in mind whenever quantitative nutritional studies, especially those involving synthetic diets, are being carried out.

c. Deposition of Lipids. (1) *Blood Vessel Walls* Apart from the usual lipids concerned in cell structure, blood vessel walls normally contain no other lipids Under certain circumstances, however, lipids may become deposited in the walls of the blood vessels, giving rise to atheroma. These degenerative changes are commoner in older people and lead to severe cardiovascular disease.

The lipids present in these deposits are essentially glycerides and cholesterol It has been shown by the use of labeled cholesterol that this mate-

[403] W Crampton, R H Common, F A Farmer, Berryhill, F. M, and L Wise-

[404]

[405] . .

[406] . . itinal absorption, with special reference to the products of rancidity, Ph D. Thesis, University of Birmingham, 1949

CHAPTER 8

The Fat-Soluble Vitamins

(With Special Reference to the Requirements of Different Animals)

THOMAS MOORE

Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council

I. General Introduction

Different species of animals vary considerably in their requirements for fat-soluble vitamins and in their abilities to store or metabolize these

disease is not uncommonly associated with coronary disease and that some alteration in cholesterol metabolism, and the stability of bound cholesterol might form a common link between them.

In conclusion, it is clear that there is a continual demand for lipids in the body which is met by ingestion and by biosynthesis A number of essential dietary constituents such as polyethenoid fatty acids, choline, thiamine, and pyridoxine are important for normal lipid metabolism. In most animals a balanced intake of carbohydrate, protein, and lipids is probably best However, considerable variations are found and imbalance can be corrected by biosynthesis, provided that the necessary starting materials that cannot be synthesized in the body are available and that conflicting priorities for biosynthesis do not arise.

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I. General Introduction

Different species of animals vary considerably in their requirements for fat-soluble vitamins and in their abilities to store or metabolize these

vitamins in their tissues. These divergencies may be both qualitative and quantitative, and they may reflect profound differences in the paths of metabolism or merely minor differences in the efficiencies of absorption and utilization.

In some instances one animal may require a vitamin which is unnecessary for another Thus vertebrates require vitamin A, which they may obtain either by eating food of an animal origin or by the conversion of a carotenoid provitamin of vegetable origin in their own bodies. The tissues of many invertebrates, on the other hand, remain devoid of vitamin A, even when large amounts of provitamins are present in the diet. As examples of a less fundamental degree of distinction we have many instances of the same vitamin being required by different species, but in widely divergent amounts It has been clearly shown, for example, that chicks may be made deficient in vitamin K by giving them diets which would be quite innocuous to rats

In deficiency of the same vitamin, moreover, the lesions which are most commonly incurred in different animals may vary considerably. In rats and several other animals deprivation of vitamin A is often first indicated by xerophthalmia, but in pigs the earliest sign of abnormality may be an inability to stand on the hind legs. Finally, the existence of all the fat-soluble vitamins, A, D, E, and K, in more than one form and the multiplicity of carotenoid pigments capable of acting as provitamins A introduce a further complication Alternative forms of the same vitamin, such as vitamins D_2 and D_3, when supplied to the rat and chick, may not always be equally effective in different animals.

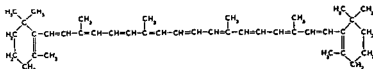
II. Vitamin A

Preformed vitamin A has the distinction of being entirely an animal product, and its relation to β-carotene and other carotenoid provitamins still presents many interesting problems. The essential chemical change, which results in the formation of vitamin A from its provitamins, appears to consist of the addition to the carotenoid of 2 molecules of water to give 2 molecules of the vitamin, $C_{20}H_{30}O$, by central fission Recently Meunier[1] *has found that β-carotene, the most important provitamin, may* be oxidized to vitamin A aldehyde, $C_{20}H_{28}O$, by adsorption on manganese dioxide, and Glover, Goodwin, and Morton[2] have shown that the aldehyde is readily reduced to vitamin A by the rat. It seems probable, therefore, that the conversion may involve oxidation and subsequent reduction of the carotene molecule rather than the addition of water in a single stage.

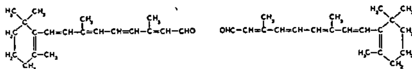
[1] P. Meunier, *Intern. Z. Vitaminforsch* **23**, 21 (1951)
[2] J. Glover, T. W. Goodwin, and R. A. Morton, *Biochem J.* **43**, 109 (1948)

1 Carotenoid Metabolism in Plants

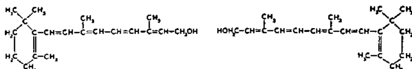
On paper these changes appear simple, and it is perhaps remarkable that no plant has yet been found which is capable of deriving vitamin A from the provitamins which occur so abundantly in green and yellow vegetable tissues Enzymes capable of decolorizing carotene are known to occur in some plant tissues and have been extensively studied in the soy bean.[3] In this instance, at least, the effect is due not to an enzyme acting specifi-



β-Carotene



Two molecules of retinene, or vitamin A aldehyde, as obtained by the oxidation of β-carotene on manganese dioxide



Two molecules of vitamin A, as obtained from retinene by reduction *in vivo* or by chemical means

cally on carotenoids but to a lipoxidase which attacks the carotene through the medium of oxidized unsaturated fatty acids

Many workers in the field must have been impressed by the widely different distributions of carotenoids between closely allied vegetable tissues Thus most carrots are strongly yellow, whereas parsnips and a few varieties of carrots are almost white These variations may be due to differences in the oxidation systems of the various plants, or possibly to different modes of transporting and storing carotenoids which are produced in the leaves

2 Carotenoids in Invertebrate Animals

In a recent review Goodwin[4] has summarized the wide literature on the distribution of carotenoid pigments in marine invertebrates, including

[3] R J Sumner, *J. Biol Chem* **146**, 215 (1942)

[4] T W Goodwin, *Biol Revs Cambridge Phil Soc* **25**, 391 (1950)

anemones, worms, sea urchins, crustaceans, and various mollusks He draws attention to the frequent concentrations of carotenoids in the sexual organs and to the diversity of the pigments which are often present in the same species Thus in the gonads of the limpets *Patella vulgata* and *Patella depressa* Goodwin and Taha[5] found β-carotene, echinenone, cryptoxanthin, and zeaxanthin Astaxanthin, 3,3′-dihydroxy-4,4′-diketo-β-carotene, is very characteristic of the crustaceans, although not completely confined to them

Until Wald's work on the presence of vitamin A in the eyes of crustaceans, which will be mentioned later, there was little evidence that the provitamins A, such as the carotenes, cryptoxanthin, and echinenone, were any more indispensible than the non-provitamins, such as the xanthophylls and astaxanthin, in the metabolism of invertebrates. In the locust, according to recent studies by Goodwin,[6] there is even evidence that a provitamin may be converted to a non-provitamin Thus β-carotene was found to be the only pigment originally present in the fertilized eggs, but after incubation astaxanthin made up 70% of the carotenoid pigments then present.

3. Carotenoids in Vertebrate Animals

Vitamin A, either as vitamin A_1 or as the less familiar vitamin A_2, appears to be present in all vertebrates Great differences are found, however, in the efficiency with which provitamins are utilized and stored as vitamin A, and in the carotenoid pigments which accompany the vitamin in the tissues Many animals, including the sheep, the goat, the pig, the rat, the guinea pig, and most rabbits have almost completely white body fat and show only small or negligible amounts of carotenoids in their blood and tissues The virtual absence of carotene in the tissues of these animals agrees well with the generally accepted view that its conversion to vitamin A takes place in the tissues of the small intestine. It must be assumed that pigments which accompany carotene in the diet are either not absorbed from the intestine or are degraded in the tissues into colorless products which do not share with vitamin A the ability to give color reactions with the antimony trichloride.

In other animals, such as man, and the cow, the horse, the hen, the frog, and the salmon, substantial amounts of carotenoids are absorbed unchanged from the intestines In the cow the main pigment coloring the blood and tissues is carotene, in the hen xanthophyll, in the salmon astaxanthin, and in man carotene usually predominates in a mixture which generally also contains some xanthophyll and a small amount of lycopene, together with pigments as yet unidentified It might be surmised that the presence of substantial amounts of carotene in the tissues would indicate that the

[5] T W. Goodwin and M M Taha, *Bio*
[6] T W Goodwin, *Biochem J* 45, 472

conversion of carotene to vitamin A was less efficient than in a white-fatted animal The general superiority of the vitamin A reserves of the sheep over those of the cow (see below) would support this view, but the white-fatted guinea pig is usually particularly inefficient in converting carotene

Even in similar animals, or in different breeds of the same species, there may be remarkable differences in the amounts of unchanged carotene which are present in the tissues Cows of the Jersey and other Channel Island breeds have highly pigmented fat, and their serum often contains 2000 μg or more of carotene per 100 ml In other breeds 200 μg might be considered a typical level during summer when the intake of carotene is high The fat of the buffalo, another animal of the same genus, is reported to be colorless

TABLE 1

STORAGE OF VITAMIN A IN THE LIVERS OF VARIOUS ANIMALS

	I u /g		
Guinea pig	10	↑	0
Pig	100		↑
Cow	150		
Rabbit	170	Normal human	
Rat (wild)	250	(median 320)	
Sheep	600		Exp rat
Codfish	2,000	↓	
Sperm whale	4,400		
Bearded seal	13,000		
Polar bear	20,000 (toxic)		↓

4 LIVER STORES OF VITAMIN A

The concentrations of vitamin A stored in the liver are influenced by the species of the animal and by its diet High concentrations may be attained in the livers of all the more familiar animals which have so far been investigated by giving them large doses of preformed vitamin A Values for any particular species, therefore, can be considered to be typical only when they are related to a characteristic nutritional background A selection of such typical values is given in Table 1

An interesting comparison may be made between the figures for the rabbit and guinea pig, which in both instances refer to animals kept in captivity with adequate supplies of green vegetables and examined by the author The guinea pig seems to be much less efficient than the rabbit in converting carotene to vitamin A, although it is capable of accumulating large stores when given the preformed vitamin [7] The values for the cow

[7] L S Bentley and A F Morgan, *J Nutrition* 30, 159 (1945)

and the sheep also refer to animals which for long periods of their lives must have received large amounts of carotene in the form of grass, and it is interesting that concentrations in the sheep should be consistently higher than in the cow.

Very high reserves are found in polar bears and sometimes in seals.[8] It may be surmised that the seal preys upon the cod and other fish, and that the bear preys upon seals. The high concentration of vitamin A in the bears livers is probably the cause of its toxicity, which has troubled unwary Arctic explorers since Elizabethan times. The richness of whales' liver in vitamin A has been explained by Kon and Thompson[9] on the basis of the preformed vitamin which is present in the krill, or shrimps, upon which these animals subsist

The commercial importance of vitamin A has stimulated a profusion of investigations on its distribution in marine sources. The wide divergencies between concentrations in different species, however, are still hard to explain. Can the disparity between the soup-fin shark, with a liver oil containing up to 90,000 i.u of vitamin A per gram, and the basking shark with only 10 i.u. per gram, be sufficiently explained by differences in their respective diets? Or must we look for some subtle differences in metabolism which are as yet completely unappreciated?

5. The Distribution of Vitamin A in the Body

In mammals and in birds the liver under normal nutritional conditions appears invariably to be the main site of storage of vitamin A, with much smaller concentrations in the kidney, the lungs, the adrenals, the adipose tissues, and the blood plasma The eyes, in which the vitamin plays a prominent physiological role, never contain more than a minute fraction of the total reserves of the body under normal circumstances. In fishes also the liver is usually rich in the vitamin, but Lovern, Edisbury, and Morton[10] found that in the halibut substantial amounts may be present in the intestinal tissues In the hake Grangaud, Massonet, and Larroque[11] have found, indeed, that the usual predominance of the liver is often superseded by the intestines. Edisbury, Lovern, and Morton[12] found that in eels a high proportion of the body's reserves of vitamin A is located in the fat deposits

In crustaceans the distribution of vitamin A is sometimes very different from that found in vertebrates The amounts of vitamin in the eye appears

8 [illegible]

9 [illegible]

10 [illegible]

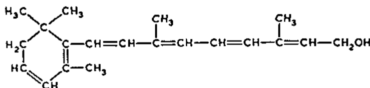
11 [illegible] (1949)

12 J R Edisbury, J. A. Lovern, and R A Morton, *Biochem J* 31, 416 (1937).

to be larger and to constitute a much higher percentage of the total vitamin A contents of the whole animal Thus Fisher, Kon, and Thompson[13] have shown that in shrimps the entire vitamin A contents of the whole organism are sometimes present in the eyes.

6 Fresh-Water Fish

The livers of fresh-water fish differ remarkably from those of salt-water fish and mammals in containing vitamin A_2 in place of, or in addition to, vitamin A_1 The presence of this interesting modification of the vitamin was recognized by spectrographic methods[14, 15] In the ultraviolet region vitamin A_2 has a maximum at about 350 mμ as compared with 328 mμ for vitamin A, while in the antimony trichloride reaction the maxima are at 693 mμ and 617 mμ, respectively Sources of vitamin A_2 free from vitamin A_1 are not readily obtained, and the isolation of pure vitamin A_2 proved a difficult project Salah and Morton,[16] however, devised an ingenious method in which the two forms were readily separated chromatographically after oxidation to the corresponding aldehydes, which can be reduced back to the alcohols



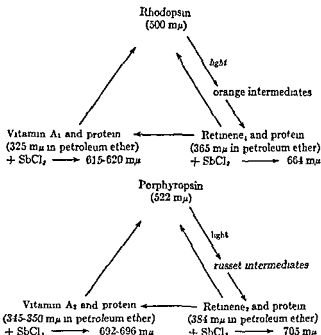
Vitamin A_2, according to Morton

When mammals are given vitamin A_2 or fresh-water fish are given vitamin A_1, the two forms of the vitamin do not appear to be readily introconverted Thus Shantz *et al*[17] found that when rats were given vitamin A_2 it appeared unchanged in their livers, blood, and retinas, although the vitamin A_1 previously available was held tenaciously Recently Morcos and Salah[18] have found that when vitamin A_1 is given to the Nile fishes *Clarias lazera* and *Tilapia nilotica*, which normally contain only vitamin A_2, it is stored in the liver without being changed

[13] L R Fisher, S K Kon, and S Y Thompson, *Biochem J* **49**, xv (1951)
[14] J R Edisbury, R A Morton, and J W Simpkins, *Nature* **140**, 234 (1937)
[15] A E Gillam, I M Heilbron, W E Jones, E Lederer, and V Pasanova, *Biochem J* **32**, 405 (1938)
[16] M K Salah and R A Morton, *Biochem J* **43**, lvi (1948)
[17] E M Shantz, N D Embree, H C Hodge, and J H Wills, Jr, *J Biol Chem* **163**, 455 (1946)
[18] S K Morcos and M K Salah, *Nature* **167**, 117 (1951)

7. The Rhodopsin and Porphyropsin Systems

In animals in which vitamin A_1 is the characteristic form the dark-adapted retina contains *rhodopsin, a red pigment which is often described* as "visual purple" An association between vitamin A and rhodopsin was inferred from the deterioration of dark adaptation which occurs in deficiency of vitamin A and from the absence of rhodopsin in the eyes of deficient rats, but the first direct evidence that vitamin A is a part of the rhodopsin molecule was due to Wald.[19] On exposure of the dark-adapted retina to



light rhodopsin is changed first to orange and yellow pigments which have absorption spectra which are sensitive to changes in pH Further exposure leads to the production of the yellow fat-soluble pigment retinene, which Ball, Goodwin, and Morton[20] have proved to be the aldehyde of vitamin A_1, together with protein

According to Wald rhodopsin may be regenerated rapidly and directly from retinene and opsin, one of the retinal proteins. Rhodopsin may also be formed more slowly from vitamin A and "opsin" by the action of enzymes prepared from the retina and pigmented layers of the eye and activated with cozymase [21]

[19] G Wald, *Nature* 136, 832 (1935)
[20] S Ball, T W Goodwin, and R A Morton, *Biochem J* 42, 516 (1948)
[21] G Wald, *Science* 109, 482 (1949)

In the porphyropsin system, as found in fresh-water fishes, cyclostomes, and certain amphibia, the modifications indicated are found.

The same enzyme system functions equally well with retinene$_1$ or retinene$_2$ as its substrate Wald draws attention to the interesting intrusion of nicotinamide, as part of reduced cozymase, into the metabolism of vitamin A, which affords yet another example of the interaction of vitamins[22]

8 Effects of Vitamin A Deficiency in Different Animals

In man defective dark adaptation is the first sign of deficiency of vitamin A, but in severe deficiency xerophthalmia is the most serious lesion Some investigators also consider that skin lesions such as phrynoderma of India and Ceylon are caused by deficiency of vitamin A, but others hold that deficiencies of water-soluble vitamins may be a more important cause Xerophthalmia is also frequently but not invariably observed in rats made

TABLE 2

	Bad dark vision	Xerophthalmia	Abscesses	Bony lesions	Paresis	Fetal deformities	Kidney infections and stones
Man	+	+					
Rat	?	+	+			+	+
Dog		+		+			
Rabbit		+		+			
Cow	+	+	+	+			
Pig					+	+	

deficient in vitamin A by experimental methods, as well as in cows, dogs, and certain other animals Other lesions which are common in various animals include xerosis of the mucous membranes throughout the body, infections of the respiratory system or urinary system, and overgrowth of the bones

It may perhaps be surmised that all animals would be liable to all the lesions caused by deficiency of vitamin A under conditions of stress which favored their occurrence. Thus xerosis of the respiratory tract might be encouraged by factors causing dryness or irritation, and bony lesions by the continuance of growth after the onset of deficiency It is interesting to note, however, how widely different the emphasis on the various effects of deficiency may be, as commonly seen in different animals

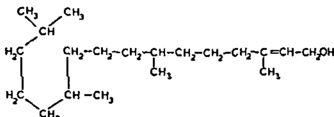
From the short list of these abnormalities given in Table 2 it will be seen that, although vitamin A is often described as an antixerosis factor, the effects of its deficiency may extend far beyond the mucous membranes and other epithelial structures Thus the best-known lesion occurring in

[22] T Moore, *Vitamins and Hormones* 3, 1 (1945)

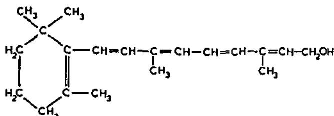
vitamin-A deficiency under field conditions in bovines is a disorganized growth of the bone, which in the skull is accompanied by a distortion and narrowing of the optic nerve which may result in blindness.[23] The same form of lesion has also been studied extensively in dogs.[24]

III. Vitamin E

From the formulas of the tocopherols it will be seen that they all consist of substituted quinones which have been condensed with the alcohol phytol. It is interesting, and doubtless of great biological significance in some way not yet fully appreciated that the same skeleton carbon chain, with at-



The alcohol phytol drawn in a twisted position to show its similarity to vitamin A.



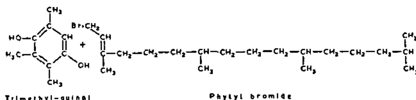
Vitamin A.

tached methyl groups, recurs in so many important biological substances Thus phytol itself is an essential part of the chlorophyll molecule and of one form of vitamin K, besides occurring in all forms of tocopherol. By desaturation and ring closure the formation of vitamin A may readily be visualized, while by the combination of two phytol molecules followed by the same modifications we may produce, at least on paper, the typical structure of the carotenoids

The classical test for vitamin E activity depends on the maintenance of pregnancy in rats given a deficient diet. Judged by this method α-tocopherol has much greater activity than the other forms, which presumably reflects the greater efficiency with which it is absorbed and stored in the tissues. In chemical experiments *in vitro* the tocopherols are found to act as antioxi-

[23] L A Moore, C. F. Huffman, and C W. Duncan, *J Nutrition* 9, 533 (1935)
[24] E Mellanby, *J. Physiol* 94, 380 (1938)

dants capable of protecting from oxidation readily oxidizable substances, such as highly unsaturated fatty acids and vitamin A Although many other substances, such as hydroquinone, may be even better antioxidants than the tocopherols *in vitro* they generally are found lacking, as Hickman and Harris[25] have pointed out, in their ability to be absorbed and stored by the tissues The tocopherols have therefore a special role as antioxidants in the animal body, as demonstrated clearly by their ability to protect the liver reserves of vitamin A[26] or to prevent the abnormalities in the fat deposits which may otherwise result from the consumption of large amounts of highly unsaturated fatty acids [27]



α-Tocopherol

Karrer's synthesis of α tocopherol from trimethylquinol and phytyl bromide

The occurrence of the phytol skeleton in vitamin E might suggest that the explanation of its power to protect vitamin A, which we have visualized as a cyclized and unsaturated form of phytol, could be found in a close similarity in the distribution of the two substances throughout the body Although both substances are equally soluble in fat, however, their distributions in the tissues are fundamentally different Vitamin A, which is present in the body mainly in the esterified form in all sites except the blood plasma, shows under normal conditions a marked preference for storage in the liver The tocopherols, which appear to be stored in the body entirely as alcohols, are more evenly distributed throughout the body, with the greatest amounts in the adipose tissues [28, 29]

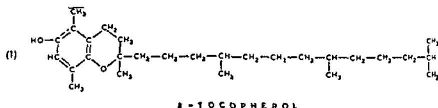
[25] K. C. D. Hickman and P. L. Harris, [illegible]

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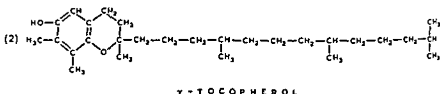
[29] M L Quaife and M Y Dju, *J Biol. Chem* 180, 263 (1949)

1. Distribution in Plants

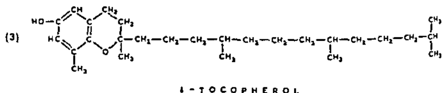
The tocopherols are present both in the foliage of plants and in seeds Until recently the wide variations in the tocopherol contents of various



β-TOCOPHEROL



γ-TOCOPHEROL



δ-TOCOPHEROL

TABLE 3

Oil	Linoleic acid, %	Total tocopherols, mg %
Coconut	2	3
Olive	6	7
Linseed	18	23
Peanut	24	36
Rice bran	37	91
Maize oil	44	110
Cottonseed	49	110
Wheat germ	50	270
Soybean	63	70

oils have been correlated with no other factor, but Hove and Harris[30] have made the interesting suggestion that the concentrations of tocopherol and linoleic acid run roughly parallel. The values in Table 3 have been extracted from the data which they have quoted in favor of their view. The

[30] E L Hove and P L Harris, *J Amer Oil. Chemists Soc* **27**, 405 (1951)

correlation is imperfect but statistically significant The authors point out that the degree of unsaturation of oils cannot in itself be taken as an indication of their linoleic acid and tocopherol contents. Thus cod-liver oil, although highly unsaturated, is reported to contain neither linoleic acid nor tocopherol Linseed oil is held to owe its high unsaturation more to the trebly saturated linoleic acid than to linolenic, and the tocopherol content is correspondingly low

2 Vitamin E in Invertebrates

The interesting work of Fraenkel and Blewett[31] has shown that both tocopherol and linoleic acids are important for the development of the larvae of moths of the genus *Ephestia* As a starting point for their investigations they observed that the larvae could be grown on artificial diet provided that wheat germ was given When only the unsaponifiable fraction of the oil was allowed, the larvae were able to grow, but the moths emerged with wings lacking in scales With the saponifiable fraction only, growth was poor but wing development and the ability to emerge were normal The unsaponifiable fraction could be replaced by α-tocopherol, and the saponifiable fraction by linoleic acid In contrast to the specificity of the tocopherols in mammalian metabolism, however, it was found that the unsaponifiable of wheat germ oil was rather more active than would have been expected from its tocopherol content Ethyl and propyl gallates, moreover, could replace tocopherol, linolenic acid could replace linoleic Cod-liver oil, rich in docosahexenoic and other higher unsaturated acids caused rapid growth, but poor wing development and emergence

3 Vitamin-E Deficiency in Mammals

Mason[32] and Moore[33] and others have commented on the multiplicity of the structural and functional changes induced by deficiency of vitamin E Infertility may occur either through resorption of the fetuses[34] or through testicular degeneration [35] The uterus, even in virgin animals, may develop a characteristic brown pigmentation, which later extends to the skeletal muscles [36] In the adipose tissues a somewhat similar, though not identical form of pigmentation has also been observed [37] Muscular dystrophy is very common [38] Exudative diathesis,[39] encephalomalacia,[40] renal abnormali-

[31] G Fraenkel and M Blewett, *J Exptl Biol* **22**, 172 (1951)
[32] K E Mason, *Vitamins and Hormones* **2**, 107 (1944)
[33] T Moore, *Brit J Nutrition* **2**, 407 (1949)
[34] H M Evans and G O Burr, *Mem Univ Calif* **8**, 1 (1927)
[35] H M Evans, *Proc Natl Acad Sci U S* **11**, 373 (1925)
[36] A J P Martin and T Moore, *J Soc Chem Ind (London)* **55**, 236 (1936)
[37] H Dam and H Granados, *Science* **102**, 327 (1945)
[38] H S Olcott, *J Nutrition* **15**, 221 (1938)

ties,[41, 42] ceroid pigmentation in the liver,[43] and the uneconomical use of protein,[44] and of vitamin A[45] have been reported.

These lesions are remarkable, moreover, not only for their wide variety but for their different incidence between species. Failure of reproduction may readily be demonstrated in rats, but herbivora appear to be much more resistant Brown pigmentation of the uterus is also characteristic of deficiency in the rat, but although other species also show pigmentation their uteri do not appear to be specially affected. Adult rabbits may be made to develop severe muscular dystrophy by deficiency of vitamin E for a few weeks, with creatinuria as a plain biochemical indication of their lesion, which may be cured by the administration of tocopherol. Dystrophy also appears rapidly in weanling rats, reared by mothers given marginal doses of tocopherol, but older animals may sometimes subsist on a deficient diet for more than a year without the development of the paresis which gives an external indication of dystrophy. Exudative diathesis and encephalomalacia have been described almost exclusively in chicks.

As a further complication it is well established that the development of several of the abnormalities caused by deficiency of vitamin E may be influenced by other components of the diet. These include the amount, character, and degree of unsaturation of the fat, the protein allowance, and all the participants in the complicated lipotrophic systems, including choline, cystine, methionine, inositol, and cholesterol, which are responsible for the prevention of the excessive deposition of fat in the liver. The result is an intricate network of interrelationships, which will be fully clarified only by prolonged research. An attempt to suggest graphically the complexity of these interrelationships is made in Fig. 1.

IV. Vitamin D

The formation of D vitamins by the ultraviolet irradiation of 7-dehydrocholesterol or ergosterol is now a well-established and central fact in our knowledge of the vitamins Our first sense of wonder at such a remarkable phenomenon, therefore, may sometimes be dulled by familiarity Let us imagine, however, that an intelligent chemist, from whom *all knowledge* of the mode of action of the vitamins had been withheld, could be given a list of the chemical and physical properties of the vitamins and provitamins, and then asked to name those which would be influenced *in their action* by

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[39] H Dam and J Glavind, *Nature* **142**, 1077 (1938)

[40] H Dam, J Glavind, O Bernth, and E Hagens, *Nature* **142**, 1157 (1938)

[41] [illegible] (London) **57**, 973 (1936)

[42] [illegible]

[43] [illegible], 375 (1945)

[44] H Dam, *Proc Soc* [illegible]

[45] A W Davies and T Moore, *Nature* **147**, 794 (1941)

exposure to light. His first choice might well fall on riboflavin, with its yellow color and vivid greenish yellow fluorescence. Secondly he might guess that light was concerned in the conversion of the intensely yellow carotene into vitamin A. In neither case, however, have we any substantial evidence to support these suggestions. Riboflavin and carotenoids are certainly present in the retina, but we have little or no evidence that they act as photoreceptors in the general system, or that they are influenced by the visible irradiation to which the skins of most animals are at least partially exposed during daylight.

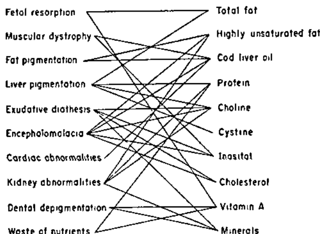


FIG. 1. Factors affecting the occurrence of lesions due to vitamin E deficiency. The diagonal lines indicate claims that deficiency or excess of the nutrient listed in the right hand column interacts with deficiency of vitamin E to cause or aggravate the abnormality in the left hand column with which the nutrient is linked. [From T. Moore, *Brit. J. Nutrition* **2**, 407 (1949).]

On receiving a hint that the effective irradiation lies beyond the visible range our chemist might first show interest in vitamin A, with its intense absorption at 328 mμ. He would learn, however, that this vitamin is located mainly in the liver, without clear physical evidence of its presence in the skin. After this further disappointment he might at last be attracted by the intricate absorption spectra of the D provitamins, with their absorption maxima at 262, 272, 282, and 294 mμ. He would thus rediscover the clue which a quarter of a century ago led Rosenheim and Webster[46] in England and Windaus and his colleagues[47] in Germany to achieve the synthesis of vitamin D_1, the first vitamin to be identified as a pure chemical compound.

[46] O. Rosenheim and T. A. Webster, *Lancet* **2**, 622 (1927).

[47] A. Windaus and A. Hess, *Nachr. Ges. Wiss. Göttingen, Math.-physik. Klasse* 175 (1927).

The D vitamins are unique in being replaceable by exposure of the skin of the subject to ultraviolet irradiation. For many years it was known that rickets in humans, a disease characterized by the defective calcification of the bones, could be prevented or cured by the inclusion in the diet of certain fats, such as cod-liver oil or butter. Alternatively the body might be exposed to sunlight or artificial sunlight in the form of ultraviolet irradiations. Steenbock and Block[48] linked together these parallel lines of advance by means of their classical discovery that *foods become antirachitic after exposure to irradiation* In a period of surprisingly rapid development it was then shown that antirachitic activity was not associated with a vague "excitation" of the molecules of the food in general or by a non-specific activation of fats For a time cholesterol, a major constituent of the non-saponifiable matter of animal fats and an important constituent of nervous tissues, was considered to be the provitamin. Purified cholesterol, however, was eventually found to be incapable of activation, and the provitamin was traced to a highly absorbing contaminant which was present in varying amounts as impurity.

1. Vitamin D_2 or Calciferol

At this point *an interesting twist in the line of progress occurred.* The same *four-banded spectra is now known to be characteristic* of several steroids, including 7-dehydrocholesterol, which occurs in animal tissues and ergosterol, which takes its name from ergot but which is also found in plants and yeast It will be seen that these two steroids differ only in the constitution of their side chains

Ergosterol is present as the main sterol in several sources, including yeast, and it had been isolated and identified before any connection with vitamin D was realized 7-Dehydrocholesterol, on the other hand, was present only as a contaminant of cholesterol in the more familiar animal materials handled by biochemists and had therefore escaped isolation *The eager* search through the chemist's shelves for a sterol showing the desired absorption spectrum inevitably focused attention on ergosterol

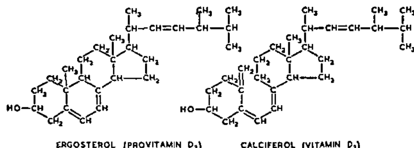
Irradiation of this unsaturated sterol produced a resin which cured rickets in rats at a level of dosing which reflected the greatly increased intensity of its absorption spectrum as compared with those of the impure forms of *cholesterol which had previously been studied The task of isolating the* vitamin from the resin, however, proved difficult and the first crystalline [illegible] isolated vitamin D [illegible] was found to contain the active prin- [illegible] vitamin D_2, however, was proved [illegible] name "calciferol"

[48] H Steenbock and A Black, *J Bio*

2 The Inefficiency of Calciferol in Chickens

In the investigations so far mentioned rats were usually employed for testing antirachitic activity Typical cod-liver oil was used as a basis for measuring the activity of irradiated impure cholesterol, and as stated above irradiated ergosterol possessed the higher activity which was to be expected from spectroscopic considerations When calciferol was tested upon chickens, however, it was found to be much less active than would have been expected from the trials with rats

Thus Massengale and Nusmeier[49] found that calciferol was about one hundred times less effective than cod-liver oil when doses equivalent in rat units were given, and many other workers obtained similar evidence of the inferiority of calciferol[50-53] The comparative biochemist is therefore provided with a striking instance of the different requirements of species,



since a minor modification from the main natural formula for vitamin D, involving only the constitution of the side chain, is of profound importance to the chick but of little significance to the rat

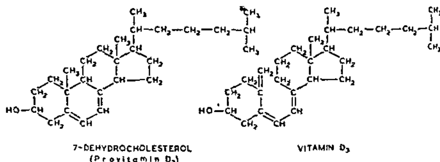
3 Provitamins in Plants and Animals

It is important to remember that there is a fundamental difference in the sense in which the word "provitamin" is used in relation to the carotenes and other provitamins A and to 7-dehydrocholesterol, which functions as the main natural provitamin D The carotenes cannot be synthesized in the animal organism, but once they have been ingested they are converted to the vitamin without external aid On the other hand the animal has been proved capable of synthesizing cholesterol, and can presumably

[49] O N Massengale and M Nusmeier, *J Biol Chem* 87, 423 (1930)
[50] F E Mussehl and C W Ackerman, *Poultry Sci* 9, 334 (1930)
[51] H S Steenbock, W F Kletzien, and J G Halpin, *J Biol Chem* 97, 249 (1932)
[52] R M Bethke, P R Record, and D C Kennard, *J Nutrition* 6, 413 (1933)
[53] W C Russell M W Taylor, and D E Wilcox, *J Nutrition* 9, 569 (1935)

convert it to 7-dehydrocholesterol, but external aid is required, in the form of irradiation, to convert this substance into the active vitamin.

An interesting exploration of the distribution of ergosterol and other precursors of vitamin D, as indicated by the absorption spectra of crude sterols isolated from various sources, was made in 1936 by Gillam and Heilbron.[54] Cholesterol that had been prepared from brain contained 0.01 to 0.04% of provitamins, estimated as ergosterol. Cholesterol from fish-liver oil contained 0.1%, and from egg yolk 0.22%. Sterols from cod and herring roes contained 0.35 and 0.55%, respectively, and from marine plankton 0.35%. The highest concentrations in animals were found in low forms of marine life, with 1.8 to 5.5% in oysters, 4.6 to 12% in lug worms, and 16.7 to 10.6% in sea anemones. Vegetable sterols obtained from cocksfoot grass contained 0.81% of spectroscopically absorbing sterol, and from perennial rye grass 1.48%.



More recently Scott, Glover, and Morton[55] have found as much as 6% of 7-dehydrocholesterol in the unsaponifiable matter from the intestines of guinea pigs, and about 0.5% in similar extracts from rats and oxen. They conclude that the intestines contain an enzyme which is capable of maintaining the balance between cholesterol and 7-dehydrocholesterol. There is also some evidence that the provitamins play some part in the sex processes. Thus Morton and Rosen[56] have observed high concentrations of 7-dehydrocholesterol in the mature ovaries of frogs. Moore and Ward[57] have noticed that 7-dehydrocholesterol is present in high concentrations in certain parts of the genital system of male rats, such as the caput epididymis and preputial glands, but not in others, such as the testes, cauda epididymis,

[54] A. E. Gillam and I. M. Heilbron, *Biochem. J.* 30, 1253 (1936).
[55] M. Scott, J. Glover, and R. A. Morton, *1st Intern. Conf. Biochem.* Hepworth Tunbridge Wells, 1949, p. 10.
[56] R. A. Morton and D. G. Rosen, *Biochem. J.* 45, 612 (1949).
[57] T. Moore and R. J. Ward, *Biochem. Soc. Symposia*, No. 7, p. 33 (1951).

seminal vesicles, coagulating glands, and prostate So far they have failed to observe the same phenomenon in any other animal [58]

4 The Conversion of 7-Dehydrocholesterol to Vitamin D_3

The artificial preparation of 7-dehydrocholesterol from cholesterol and its irradiation to produce vitamin D_3 was accomplished by Windaus and his colleagues [59] There is now no difficulty, therefore, in producing artificially the form of vitamin D which is specially required by poultry

It might be thought, therefore, that with evidence of the production of 7-dehydrocholesterol in the animal, and of its ready conversion to the natural vitamin D by irradiation it would be a simple matter to deduce some broad general scheme which would cover the formation and storage of the vitamin in various species In practice further information is still required on many puzzling points

5 Marine Plankton

Near the surface of the sea the plankton are exposed to intense sunlight The discovery by Steenbock and Black that foods may be rendered antirachitic by irradiation therefore suggested that vitamin D might arise mainly in the minute plankton and be transferred first to small and then to larger fish, sharks, seals, and other predatory animals Experiments by Leigh-Clare,[60] however, failed to demonstrate any vitamin D in the marine diatom *Nitzschia closterium*, and equally negative results were found by Drummond and Gunther[61] for both phytoplankton and zooplankton

In zooplankton collected during spring Belloc, Farbre, and Simonnet[62] claimed to have detected ergosterol but no vitamin D, but in specimens collected during summer vitamin D was possibly present Darby and Clarke[63] studied the problem in clear water near the Tortugas Islands, where they found by spectrographic methods that ultraviolet irradiation penetrated to a depth of 3 ft Extracts made from sargassum weed were found to have definite antirachitic activity

6 Fish and Sharks

Bills[64] has commented on the wide range of vitamin D contents found in fish oils In a long table he has included a value of nil for sturgeon liver,

[58] T Moore and R J Ward, unpublished results (1951)
[59] A Windaus, H Lettre, and Fr Schenck, *Ann* **520**, 98 (1935)
[60] J L Leigh Clare, *Biochem J* **21**, 368 (1927)
[61] J C Drummond and E R Gunther, *Nature* **126**, 398 (1930)
[62] G Belloc, R Farbre, and H Simonnet, *Compt rend* **191**, 160 (1932)
[63] H H Darby and H T Clarke, *Science* **85**, 318 (1937)
[64] C E Bills, *Physiol Revs* **15**, 1 (1935)

10 i u per gram for haddock liver oil, 100 i u for cod liver, 1,300 i u. for halibut liver, 10,000 i.u. for swordfish liver, and 40,000 i u. for bluefin tuna liver

It is difficult to explain these differences either on the basis of diet or of exposure to sunlight. Thus basking sharks subsist on plankton and spend hours lying on the surface of the ocean, but their livers contain very little vitamin D. Some workers[65, 68] have noticed that the liver is often low in vitamin D in fishes with imperfect bone formation, such as sharks, dogfish, skates, rays, and lampreys. It is evident, however, that no sharp distinction can be drawn between cartilaginous and hard-bone fishes; as stated above, the liver oil of the sturgeon, a true fish, is very low in vitamin D.

Bills[69] found that the capelin, upon which the Newfoundland codfish feeds, contains only about 3 i.u. of vitamin D per gram of its whole body In experiments with young catfish he obtained some evidence that vitamin D could be synthesized by the fish themselves without the aid of light Although they were sensitive to ultraviolet irradiation it had no effect in increasing their vitamin D contents.

7. Birds

The Chinese worker Hou[70-73] made intensive studies on the mode of formation of absorption of vitamin D in birds The preen gland, or glandula urapygialis, secretes an oil, presumably containing a provitamin, which the bird transfers to its beak before preening its feathers. After exposure to the sun the vitamin D is either ingested by the mouth during subsequent preening or absorbed by the skin

In support of these conclusions the feathers and skin of normal birds were found to be antirachitic, but the same materials from either rachitic birds or birds from which the preen glands had been removed were inactive Removal of the preen glands increased the susceptibility to rickets and prevented the beneficial action of irradiation.

8 Mammals

In mammals the absorption of antirachitic substances from the skin by the oral route must obviously have less general significance It has been claimed that rachitic rats are not cured by irradiation when they are

65 [illegible] 74, 648 (1930)
66 [illegible] 189, 229 (1930)
67 [illegible])
68 [illegible]
69 [illegible]
70 H C Hou, *Chinese J. Physiol* 2, 345 (1928)
71 H C Hou, *Chinese J Physiol* 3, 171 (1929)
72 H C Hou, *Chinese J. Physiol* 4, 79 (1930)
73 H C Hou, *Chinese J. Physiol* 5, 11 (1931)

prevented from licking their fur,[74] and in this connection it is interesting to remember the extremely high concentration of 7-dehydrocholesterol in the preputial glands It seems hardly probable, however, that the human inhabitants of tropical countries enjoy freedom from rickets only through an unsuspected indulgence in skin licking Direct absorption of the vitamin into the system from the skin was supported by the observations of Hess and Weinstock[75] that irradiated skin is antirachitic and that irradiated sterol is effective when administered subcutaneously

An interesting problem is raised by the general paucity of mammalian livers in vitamin D Even the whale, which accumulates enormous concentrations of vitamin A in its liver, seems to adhere to this rule It appears probable that the storage of both vitamins A and D depend upon metabolic factors no less than upon the amounts of vitamins and provitamins available in the diet

9 Vitamin D Deficiency in Different Animals

More adequate explanation also seems desirable on the great difference in the susceptibility of different species to rickets Thus human children, dogs, and poultry are prone to rickets when subsisting upon any diet which is deficient in vitamin D, although their susceptibility may certainly be influenced by dietary modifications, such as the presence or absence of cereals containing phytic acid Some types of disease, moreover, may give rise to conditions under which enormous doses of vitamin D are necessary for the curing of rickets [76, 77] In contrast the experimental rat remains free from rickets unless it is both deprived of vitamin D and given a diet in which the balance between calcium and phosphorus is made grossly abnormal, usually by giving great excess of calcium The young from mothers who have been dosed too liberally with cod-liver oil are usually of no value for vitamin D estimations

V. Vitamin K

Two natural forms of the antihemorrhagic vitamin K are known The most important sources of vitamin K_1, which is a phytyl derivative of 2-methyl-1,4-naphthoquinone, are green plant tissues or tissues which have passed through a green stage Thus ripe tomatoes and leaves that have turned yellow retain their vitamin K_1 contents. Vitamin K_2, in which a longer farnesyl side chain replaces phytol, differs remarkably in being a bacterial product present in sources such as feces and putrefied fish meal

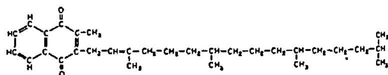
[74] E Rekling, *Strahlentherapie* **25**, 568 (1927)

[75] A F Hess and M Weinstock, *J Biol Chem* **64**, 181 (1925)

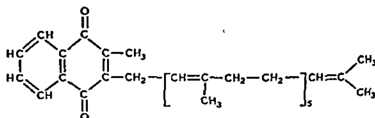
[76] F Albright, A M Butler, and E Bloomberg, *Am J. Diseases Children* **54**, 529 (1937)

[77] R A McCance, *Quart. J. Med* **16**, 33 (1947)

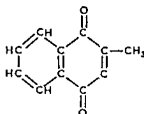
Both of these natural vitamins act by stimulating the formation of prothrombin According to Dam[78] and others prothrombin in the presence of calcium and of thromboprotein derived from tissues or blood platelets gives rise to the enzyme thrombin, which effects clotting by converting fibrinogen to fibrin Apparently the formation of prothrombin, which is measured by



Vitamin K_1
(2-Methyl-3-phytyl-1,4-naphthoquinone)



Vitamin K_2
(2-Methyl-3-difarnesyl-1,4-naphthoquinone)



Menadione
(2-Methyl-1,4-naphthoquinone)

. . . animals
added
ective
when given to the intact animal as a source of vitamin K.

. . . Norstedt &

. *Physiol*

79, 121 (1938).

1 The Activity of 2-Methyl-1,4-naphthoquinone

Since the splitting up of vitamin E into methylated quinone and phytol involves loss of biological activity, it might be expected that the removal of the side chains of the natural K vitamins would have the same effect All workers are agreed, however, that 2-methyl-1,4-naphthoquinone, without a side chain, is fully as potent as the natural forms. For therapeutic purposes, therefore, the simple artificial naphthoquinone, known as menadione, is preferred to the natural vitamins

2 Vitamin K Deficiency in Different Species

The existence of a vitamin necessary for blood coagulation was discovered by Dam in the course of experiments on chicks In an investigation with his colleagues[80] he later studied the susceptibility of other animals to deficiency. Ducklings and goslings were as vulnerable as chicks, but only mild hypoprothrombinemia occurred in pigeons and canaries Rats, guinea pigs, and dogs could be reared for long periods on deficient diets without showing any ill effects

The particular liability of chicks and other birds to deprivation of vitamin K has been explained by the relative shortness of their large intestines, which prevents adequate intestinal synthesis Dam and Glavind[81] have reported that when rats are maintained on a defective diet for long periods a proportion of the animals at length develop hypoprothrombinemia They consider that the irregular incidence is due to differences in the intestinal flora between individuals. Raoul and Ragher-Hanna[82] observed hypoprothrombinemia in rats given sulfadiazine to suppress bacterial growth in the intestines together with paraffin to prevent the absorption of vitamin K.

In the human, dosing with 2-methyl-1,4-naphthoquinone has been found to be effective in combatting hemorrhage in newborn babies[83-86] and in obstructive jaundice and other conditions leading to defective fat absorption[87-90]

[80] H Dam, F Schønheyder, and L Lewis, *Biochem J.* 31, 22–27 (1937)

[81] H Dam and J Glavind, *Z Vitaminforsch* 9, 71 (1939)

[82] Y Raoul and N Rogher-Hanna, *Bull soc chim biol* 30, 648 (1948)

[83] W W Waddell, D Guerry, and O R Kelley, *Proc Soc Exptl Biol* 40, 432 (1939)

[84] K K Nygaard, *Acta Obstet Gynecol Scand* 19, 361 (1939)

[85] H Dam, E Tage-Hansen, and P Plum, *Lancet* 2, 1157 (1939)

[86] A J Quick and A M Grossman, *Proc Soc Exptl Biol* 40, 647 (1939)

[87] E D Warner, K M Brinkhous, and H P. Smith, *Proc Soc Exptl Biol* 37, 638 (1938)

[88] H M Butt, A M Snell, and A. E Osterberg, *Proc Staff Meetings Mayo Clinic* 14, 407 (1938)

[89] H Dam and J. Glavind, *Lancet* 1, 720 (1938)

3. Antivitamins K

No account of vitamin K would be complete without mention of the anticoagulant 3,3'-methylenebis-4-hydroxycoumarin, generally known as dicoumarin. This substance was found by Link[91] to be the cause of the poisoning of cattle by spoilt sweet clover It also opposes the action of vitamin K in other animals and has been given trials in human medicine in the prevention or cure of thrombosis. Its action may be neutralized by sufficiently heavy doses of menadione, but for this purpose natural vitamin K, is more effective.

Numerous artificial substances also possess various degrees of activity either as vitamins K or antivitamins K.

VI. Essential Fatty Acids

The important investigation of Burr and Burr[92] established that rats could not thrive on diets rigidly freed from fat They observed failure in growth, kidney lesions, scaliness of the skin, and a characteristic necrosis of the tail Later they found that the lesions could be cured by the administration of linoleic and certain other polyethylenic acids, but not by oleic acid or saturated acids.[93] The indispensibility for the rat of "essential fatty acids" was therefore recognized, and they are sometimes designated vitamin F

The indispensibility of essential fatty acids to different species has not been widely studied, possibly on account of the difficulties involved in obtaining diets free from fats. In dogs kept on a diet free from fat for 3 months, however, Hansen and Wiese[94] noticed that the skin became scurfy, and the effectiveness of the diet was indicated by a fall in the iodine value of the blood fatty acids as compared with those of control animals Restriction of human subjects to a diet low in fats was found by Burr and his colleagues[95, 96] to have no ill effects, but rather a beneficial influence in reducing fatigue and, in one subject, preventing migraine. The possibility that certain skin abnormalities in the human may be associated with deficiency of fatty acids has been studied by several investigators, but the results have so far been inconclusive [97-102]

[90] J M MacFie, A L Bacharach, and M R A Chance, *Brit Med J* **2**, 1220 (1939)

[91] K P Link, *Federation Proc* **4**, 176 (1944)

[92] G O Burr and M M Burr, *J Biol Chem* **82**, 345 (1929)

[93] G O Burr and M M Burr, *J Biol Chem* **86**, 587 (1930)

[94] A E Hansen and H F Wiese, *Proc Soc Exptl Biol* **52**, 203 (1943)

[95] W. R. Brown, A E Hansen, I McQuarrie, and G. O Burr, *Proc Soc. Exptl Biol* **36**, 281 (1937)

[96] W R. Brown, A E Hansen, G O Burr, and I McQuarrie, *J. Nutrition* **16**, 511 (1938)

[97] T. Cornbleet and E R. Pace, *Arch Dermatol Syphilol* **31**, 224 (1935)

The requirement of moths for "essential fatty acids" has already been mentioned in the section on vitamin E

VII. Conclusions

The time is not yet ripe to attempt to formulize any unified scheme which will give parts to all the fat-soluble vitamins in maintaining the health of the organism Quite possibly these various vitamins, although sharing the common property of insolubility in water, may act mainly through independent channels and have relatively little interplay We have good evidence, however, that vitamin E may intervene in the protection of vitamin A Vitamins A and D, moreover, are both concerned in the formation of bones, although in completely different ways The degree of chemical similarity between vitamins E and K might suggest the possibility of some connection between their mode of action Our only hints of the reality of such an interrelationship must be drawn from claims by Shute and his colleagues[103] that vitamin E is valuable in treatment of thrombosis in leg ulcers, and the finding by Wooley[104] that α-tocopherylquinone has hemorrhagic effects in mice which may be counteracted by vitamin K

It may perhaps be of interest to emphasize certain points of difference in the metabolism of the fat-soluble vitamins, most of which have already been mentioned in this review The greatest efficiency of storage is found with preformed vitamin A, and this factor is preferentially absorbed by the liver to a much greater extent than the other vitamins With vitamin D, however, concentration in the liver occurs in fishes, but not in mammals Before vitamin A is stored in the liver it is esterified, even if it has been administered as the free alcohol, but vitamin E is stored in the tissues as the free alcohol even if administered as an ester Our knowledge of such relationships in regard to vitamins D and K is limited Vitamin D_3 seems to be unique in being derived from a provitamin which can be synthesized in the animal

The natural K vitamins, as we have seen, are peculiar in preserving their activity after the splitting off of their massive side chains If it were proved that they normally undergo fission in the course of exerting their

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[98] S J Taub and S J Zakon, *J Am Med Assoc* 105, 1675 (1935)

[99] H K Faber and D B Roberts, *J Pediat* 6, 490 (1935)

[100] A E Hansen, *Am J Diseases Children* 53, 933 (1937)

[101] J E Ginsberg, C Bernstein, and L V Iob, *Arch Dermatol Syphilol* 36, 1033 (1937)

[102] C W Finnerud, R L Kesler, and H E Wiese, *Arch Dermatol Syphilol* 44, 819 (1941)

[103] E V Shute, A B Vogelsang, F R Skelton, and W E Shute, *Surg Gynecol Obstet* 86, 1 (1948)

[104] D W Wooley, *J Biol Chem* 159, 59 (1945)

physiological action, we might find a parallel with the conversion of carotene to vitamin A

Differences between species in regard to vitamin A are seen in the absence of preformed vitamin A from many low forms of animal life, in the presence of a special form of vitamin A in fresh-water fishes, in differences in the efficiency of conversion of carotene and the storage of vitamin A, and in the wide variety of lesions produced in deficiency. With vitamin E the lesions commonly seen in deficiency vary considerably between different species With vitamin D the bony lesions are more standardized, but animals vary greatly in their susceptibility. With vitamin K there are again great differences in susceptibility, which are apparently related to the suitability of the intestines, on anatomical grounds, for the bacterial synthesis of the vitamin.

CHAPTER 9

The Vitamin B Complex

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I. Introduction

1 Scope of the Chapter

It has been our aim to present on a broad basis the important biochemical, physiological, and nutritive properties of the vitamins of the B complex We stress the more general aspects and relationships rather than minutiae

of detail, and endeavor to give an up-to-date picture of, we believe, well-established findings rather than latest information about developments still in the formative stage.

Much fuller and more detailed accounts of the properties of these vitamins can be found in the textbooks of Rosenberg,[1] Williams *et al*,[2] Robinson,[3] Harris,[4] and Bicknell and Prescott[5] Reviews and articles dealing with general or particular aspects of recent progress will be found, among others, in *Annual Reviews of Biochemistry*, *Vitamins and Hormones*, *Nutrition Reviews*, and *Nutrition Abstracts and Reviews* We have not considered methods of assay of the B vitamins. Of publications giving details of such methods, we would mention those of Barton-Wright,[6] Coward,[7] György,[8] Dann and Satterfield,[9] and the Association of Vitamin Chemists[10]

The first part of this chapter is concerned with the biochemical properties of the individual vitamins and with their role in cellular metabolism In this part the biochemical functions of these vitamins, their specificity, and the properties of substances acting as antagonists are discussed

In the second part the vitamin economy of the whole animal is considered

2. Definition of a Vitamin

It is not easy to say what a vitamin is, but Dr Harris, in Chapter 2, gives a helpful definition

According to the definition a factor may be a vitamin for one species but not necessarily for another that can synthesize the factor in its body or with the help of the gut flora and therefore does not need it in the diet

With vitamins of the B complex the distinction between what can and what cannot be synthesized is by no means clear, as these vitamins are

[1] H R Rosenberg, The Chemistry and Physiology of Vitamins, Interscience Publishers, New York, 1942

[2] R J Williams, R E Eakin, E Beerstecher, Jr, and W Shive, The Biochemistry of the B Vitamins, Reinhold Publishing Corp, New York, 1950

[3] F A Robinson, The Vitamin B Complex, Chapman and Hall, London, 1951.

[4] L J Harris, Vitamins, Churchill, London, 1951

[5] F Bicknell and F Prescott, The Vitamins in Medicine, 2nd ed, Heinemann, London, 1946

[6] E C Barton-Wright, Practical Methods for the Microbiological Assay of the Vitamin B Complex and Essential Amino Acids, Ashe Laboratories, London, 1946

[7] K H Coward, Biological Standardisation of the Vitamins, 2nd ed, Baillière, Tindall and Cox, London, 1947

[8] P György (Ed), Vitamin Methods, 2 vols, Academic Press, New York, 1950-1951

[9] W J Dann and G H Satterfield, Estimation of the Vitamins Biological Symposia, Jaques Cattell Press, Lancaster, Pa, Vol 12, 1947.

[10] Association of Vitamin Chemists, Inc, Methods of Vitamin Assay, 2nd ed., Interscience Publishers, New York, 1951

formed by microbial synthesis in the gut and their availability to the host governs the animal's requirements for exogenous supplies. The availability is dependent not only on the architecture of the gut and hence on the species but also on the nature of the diet and other conditions.

3 The Vitamins of the B Complex

The nomenclature of the B vitamins has for long been rather confused, and most of them can boast of several synonyms. Those interested will consult the comprehensive list of Cheldelin[11] and the pronouncement of *the 16th Conference of the International Union of Pure and Applied Chemistry.*[12]

In this book names adopted by the American Chemical Society are used

At present the vitamin B group contains the following eleven well-characterized vitamins: thiamine, riboflavin, nicotinic acid, vitamin B_6, pantothenic acid, biotin, folic acid, vitamin B_{12}, inositol, *p*-aminobenzoic acid, and choline.

The structural formulas, some of the more important physical and chemical properties, and certain preferred methods of estimating these vitamins are listed in Table 1

II. Biochemical Properties of the Vitamins of the B Complex

1. General

It is known that *nearly every vitamin of the B complex forms* part of a coenzyme essential for the metabolism of protein, carbohydrate, or fatty acid. Many of these coenzymes have been isolated and characterized, and, as their properties are considered in detail in other chapters, they will be only briefly mentioned here

The fact that these vitamins are usually part of an enzyme system frequently means that they are not absolutely specific, for a closely related compound of the same molecular shape and dimensions may be able to fit equally well into the enzyme and, provided that the reactive groups are in the appropriate spatial positions, perform the usual function of the vitamin When, however, a related compound without the appropriate functional groups fits into the enzyme system, it prevents the vitamin itself from doing so *and thus acts as a vitamin antagonist, or antivitamin;* this type of antagonism can be overcome by excess of the vitamin. Besides those antivitamins whose structure is related to that of the vitamin they antagonize, there are others that inactivate the vitamin by combining with it or by

11 [illegible] *Nutrition Revs.* 9, 289 (1951)

12 [illegible] *re et*

destroying it Woolley[12] has published recently an authoritative account of antivitamins and other antimetabolites

Another type of vitamin analogue that has been extensively studied is the derivative of the vitamin (ester, amide, etc), or the fragment of the vitamin molecule, that can be reconverted or synthesized to the active vitamin by the microorganism or animal, and then used to form the coenzyme Animals and microorganisms differ in their ability to carry out such interconversions, so that derivatives of vitamins or fragments of vitamin molecules may be active for one species but not for another. In some instances certain ester and amide derivatives of vitamins are inactive for microorganisms, and yet animals can make use of them, presumably by being able to hydrolyze the derivatives to the free vitamins (see p 304)

2. Thiamine

a. Function. When phosphorylated on the hydroxyl group, thiamine yields the pyrophosphate—cocarboxylase This coenzyme, concerned in lactate and pyruvate metabolism, is essential for, and is found in, all forms of life.

b. Analogues. Many compounds structurally similar to thiamine have been prepared and tested for their biological activity, and many of them are active, but of the several found active only one, the compound prepared by Schultz,[14] with an ethyl group replacing the methyl group on the pyrimidine ring, was more active (for pigeons) than thiamine itself

In general, the following conditions must be satisfied to produce a compound active biologically. (*a*) the pyrimidine and thiazole rings linked by a 1- or 2-carbon atom chain, (*b*) a primary alcohol group on the side chain attached to the thiazole ring, (*c*) an amino group on the pyrimidine ring and a quaternary nitrogen atom in the thiazole ring

Most bacteria are able to synthesize thiamine, *Lactobacillus fermenti* is a noteworthy exception and for this reason is frequently used as a test organism in microbiological assays of thiamine Some yeasts and fungi require preformed thiamine, but most of these are able to combine suitably substituted pyrimidine and thiazole moieties, and a few can synthesize thiamine when supplied with either the pyrimidine or the thiazole portions

c. Antivitamins. (1) *Analogous Antagonists* (*a*) Pyrithiamine[15] and neopyrithiamine,[16] probably identical with it,[17] are compounds in which the

[13] D W Woolley, A Study of Antimetabolites, John Wiley and Sons, New York, 1952

[14] F Schultz, *Z physiol Chem* **265**, 113 (1940)

[15] A H Tracy and R C Elderfield, *J Org Chem* **6**, 54 (1941)

[16] A N Wilson and S A Harris, *J Am Chem Soc* **71**, 2231 (1949)

[17] D W Woolley, *J Am. Chem Soc* **72**, 5763 (1950)

TABLE 1

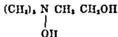
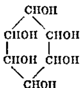
THE FORMULAS, PROPERTIES, AND PREFERRED METHODS OF ESTIMATION OF THE VITAMINS OF THE B COMPLEX

	Formula	Properties	Methods of estimation
Thiamine	CH_3 N=C NH_2 C=C·CH_2·CH_2OH Cl CH_3 C C CH_2 N N—CH CH—S HCl	White crystals, m p. 248–250° (decomp) Soluble in water and alcohol Insoluble in organic solvents. Stable in acid solution but not in alkaline	Biological rat growth; cure of bradycardia in rats Microbiological growth of *Lactobacillus fermenti*, yeast fermentation. Physical measurement of the ultraviolet fluorescence of thiochrome, produced by the oxidation of thiamine.
Riboflavin	CH_2·CH(OH) CH(OH) CH(OH) CH_2OH CH_3 N N C CO C NH CH_3 N CO	Orange crystals, m p. 282° (decomp). Slightly soluble in water (12 mg in 100 ml. at 27°) and in alcohol (4 5 mg. at 27°). Soluble in alkali but solutions deteriorate Yellow-green fluorescence in solution Absorption maxima at 221, 266, 359, and 445 mμ	Biological: rat or chick growth. Microbiological· growth of *Lactobacillus casei*. Physical measurement of ultraviolet fluorescence.

Nicotinic acid	Pyridine ring with –COOH (structure)	White crystals, m p 235–236° Soluble in water and alcohol Ultraviolet absorption maximum 385 mμ	Biological chick growth. Microbiological growth of *Lactobacillus arabinosus* Chemical color with cyanogen bromide and diazotized aromatic amines
Pantothenic acid	$CH_2OH \cdot C(CH_3)_2 \cdot CH(OH) \cdot CO \cdot NH \cdot CH_2 \cdot CH_2 \cdot COOH$	Yellow oil, soluble in water The calcium salt normally used is a white *powder*, m p 198–200°	Biological chick growth Microbiological growth of *Lactobacillus arabinosus*
Vitamin B_6	Pyridoxine (ring with CH_2OH, HO, CH_2OH, CH_3, N); Pyridoxal (ring with CHO, HO, CH_2OH, CH_3, N); Pyridoxamine (ring with CH_2NH_2, HO, CH_2OH, CH_3, N)	Pyridoxine hydrochloride, m p 206–208° (decomp) Soluble in water and alcohol	Biological rat or chick growth Microbiological growth of *Saccharomyces carlsbergensis*
Biotin	NH—CH—CH·$(CH_2)_4$·COOH; CO; S; NH—CH—CH_2 (ring structure)	White crystals, m p 230–232° Sparingly soluble in water and alcohol Soluble in dilute sodium bicarbonate	Microbiological growth of *Lactobacillus arabinosus*

TABLE 1—*Continued*

	Formula	Properties	Methods of estimation
Folic acid	Pteridine (2-amino-4-hydroxy: $N{=}C\,OH$, $H_2N\,C$, $C{-}N{=}CH$, $N{-}C{-}N{=}C$) $CH_2\,NH$ – benzene ring – $CO{\cdot}NH\,CH(COOH)\cdot CH_2\,CH_2COOH$	Orange crystals, slightly soluble in water Soluble in dilute sodium bicarbonate. Absorption maxima at 255, 282, and 365 mμ	Biological. hemoglobin measurements in chicks. Microbiological· growth of *Lactobacillus casei*.
Vitamin B_{12}	CH_2OH; $CH{-}O$; H; CH; C; $CHOH$; $O{-}P{=}O$; $O\ O$; N; CH; N; CH_3; CH_3; Co; CN; $[C_{42}H_{66}N_{9}O_{4}$ approx. $+\ 2CH_3\ CHOH\ CH_2NH_2]$	Red crystals, soluble in water. Absorption maxima at 278, 361, 525, and 549 mμ.	Biological. chick and rat growth. Microbiological growth of *Escherichia coli* or *Lactobacillus leichmannii*.

p-Aminobenzoic acid		White crystals, m p 186–187° Slightly soluble in water	Chemical: color with *p*-dimethylaminobenzaldehyde Microbiological: growth of *Lactobacillus casei*.
Choline		Colorless liquid, soluble in water and alcohol Choline chloride is normally used White crystals, hygroscopic, soluble in water	Chemical precipitation as reineckate. Microbiological growth of *Neurospora crassa*
Inositol		White crystals, m p 225–226° Soluble in water, insoluble in alcohol.	Microbiological yeast growth

thiazole ring of thiamine is replaced by a pyridine ring. Both are antagonists to thiamine for animals and bacteria.

(*b*) Oxythiamine, in which the amino group on the pyrimidine ring is replaced by an hydroxyl group, is also strongly antagonistic to thiamine for animals [18]

(*c*) *n*-Butylthiamine in which the methyl group of the pyrimidine ring is replaced by *n*-butyl, is an antivitamin for rats.[19]

(2) *Thiaminases* (*a*) Raw fish factor. The raw flesh of carp contains a factor toxic for foxes, in which it produces the so-called Chastek paralysis [20] This factor has been identified as an enzyme that can split the thiamine molecule into two parts at the methylene bridge [21] Diets containing raw fish have since been shown to produce thiamine deficiency in cats[22] and chicks [23]

(*b*) Bracken-fern factor. Air-dried bracken (*Pteris aquilina*), but not sun-dried or steamed bracken, causes in rats[24] or horses[25] symptoms of thiamine deficiency that are cured by administration of the vitamin The active agent is a thiaminase, behaving in many ways as that of raw fish [26] Bracken poisoning also occurs in ruminants though the signs are different and the condition is not cured by treatment with thiamine [27] The mode of action in ruminants is not clear. It is possible that the bracken factor exerts a profound effect on the rumen microorganisms by depriving them of thiamine and that their altered metabolism may then cause disorganization of digestive processes in the rumen, leading to deficiencies in the animal of other essential nutrients

(*c*) Live yeast Live yeast when eaten by animals or man can cause thiamine deficiency, for the yeast competes with the host for the vitamin present in the diet (see p 312)

3 Riboflavin

a. Function. Riboflavin is a constituent of two different coenzymes The first of these, flavin mononucleotide, is riboflavin-5′-phosphate. This coenzyme is concerned in carbohydrate metabolism in the oxidation of hexose monophosphate to phosphohexonic acid

[18] A J Eusebi and L R Ceredo, *Science* 110, 162 (1949)
[19] [illegible]
[20] [illegible]
[21] [illegible]
[22] [illegible]
[23] [illegible] *Soc Exptl Biol Med* 48, 376 (1941)
[24] P H Weswig, A M Freed, and J R Haag, *J Biol Chem* 165, 737 (1946)
[25] [illegible] 107, 364, 390 (1951)
[26] [illegible] xxxviii (1950)
[27] [illegible]

The second coenzyme containing riboflavin is riboflavin adenine dinucleotide, which is present in the diaphorase and xanthine oxidase enzymes This coenzyme accounts for 70 to 90% of the total riboflavin in rat tissues [28]

Apart from its occurrence in these important coenzymes, riboflavin is also present in the retinal pigment of the eye, where it plays a part in light adaptation [29]

b. Analogues. The riboflavin molecule is fairly specific Several analogues have been synthesized in which the alkyl groups attached to the isoalloxazine nucleus have been changed, and in which various pentose sugars have been substituted for D-ribose The only compounds possessing biological activity for rats were the 7-methyl-,[30] the 6-methyl-,[30] and the 6-ethyl-7-methyl-9-(D′-ribityl)-isoalloxazine,[31] and the 6,7-dimethyl-9-(L-1′-arabityl)-isoalloxazine [32]

c. Antivitamins. Several synthetic but no naturally occurring antagonists have been found These include isoriboflavin (the 5,6- instead of the 6,7-dimethyl compound), which is an antivitamin for animals but not for bacteria,[33] and the phenazine analogue (2,4-diamino-7,8-dimethyl-10-D-ribityl-5,10-dihydrophenazine), which is active in both animals and bacteria [34, 35]

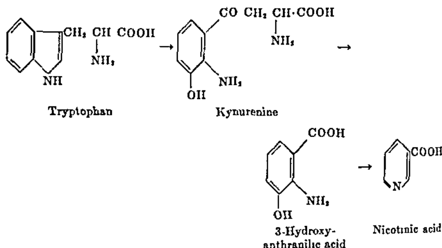
4 Nicotinic Acid

a. General. Nicotinic acid and nicotinamide are equally active as vitamins for animals and for almost all microorganisms It is of interest that animal tissues contain the vitamin almost exclusively as nicotinamide, whereas plant tissues contain it mainly in the form of nicotinic acid [36]

b. Biosynthesis from Tryptophan. During recent years it has been shown that nicotinic acid is exceptional among the vitamins of the B complex in that the rat can synthesize it within its body, the precursor being the amino acid, tryptophan [37, 38] It has now been demonstrated that this conversion of tryptophan to nicotinic acid can be accomplished by many other animals

[28] O A Bessey, O H Lowry, and R H Love, *J Biol Chem* **180**, 755 (1949)
[29] M Heimann, *Arch Ophthalmol* **28**, 493 (1942)
[30] P Karrer, H von Euler, M Malmberg, and K Schopp, *Svensk Kem Tid* **47**, 153 (1935)
[31] P Karrer and T H Quibell, *Helv Chim Acta* **19**, 1034 (1936)
[32] R Kuhn and F Weygand, *Ber* **67**, 2084 (1934)
[33] G A Emerson and J W Foster, *J Bact* **47**, 27 (1944), **48**, 97 (1944)
[34] D W Woolley, *J Biol Chem* **154**, 31 (1944)
[35] H P Sarett, *J Biol Chem* **162**, 87 (1946)
[36] W A Krehl, J de la Huerga, C A Elvehjem, and E B Hart, *J Biol Chem* **166**, 53 (1946)
[37] W A Krehl, J de la Huerga, and C A Elvehjem, *J Biol Chem* **164**, 551 (1946).
[38] H Spector and H H Mitchell, *J Biol Chem* **165**, 37 (1946).

including chicks and turkeys[39,40,41] The quantitative relationship is not simple for a variety of reasons, including the need of tryptophan as such in synthesis of body proteins, but in a general way ten to twenty parts of L-tryptophan are needed to replace one part of nicotinic acid. In chicks complete replacement is not possible.[42] Studies of the metabolism of tryptophan containing isotopic carbon by Heidelberger and his colleagues (cf ref 43) have led to the following possible scheme for the conversion:



c. Function. Nicotinamide is linked with adenine, ribose, and phosphoric acid in coenzymes I (diphosphopyridine nucleotide) and II (triphosphopyridine nucleotide) These coenzymes are dehydrogenases and are concerned with different apoenzymes, in a variety of dehydrogenation reactions, including for instance, lactate → pyruvate, glutamic acid → α-ketoglutaric acid, and retinene → vitamin A.

d. Analogues. Many compounds derived from, or closely related to, nicotinic acid possess biological activity.[44,45] In many instances the compound is active only after it has been converted into nicotinic acid (or

[39] H P. Sarett and G A Goldsmith, *J Biol Chem.* **167**, 293 (1947)
[40] R W. Luecke, W N McMillen, F Thorp, and C. Tull, *J. Animal Sci* **5**, 408 (1946)
[41] S A. Singal, A P Briggs, V P Sydenstricker, and J M. Littlejohn, *J Biol Chem* **166**, 573 (1946)
[42] J. W. West, C W. Carrick, S M Hauge, and E T. Mertz, *Poultry Sci* **31**, 479 (1952); G R Childs, C W. Carrick, and S M Hauge, *Poultry Sci.* **31**, 551 (1952)
[43] *Nutrition Revs* **8**, 85 (1950)
[44] C. A. Elvehjem and L J Teply, *Chem Revs* **33**, 185 (1943)
[45] P Ellinger, G Fraenkel, and M M. Abdel Kader, *Biochem J.* **41**, 554 (1947)

amide) by the microorganism or animal, and, as some microorganisms are unable to carry out the necessary reactions, many of the derivatives are active for animals, but not for these microorganisms

e. Antivitamins. Pyridine-3-sulfonic acid is antagonistic to nicotinic acid for microorganisms[46] but not for animals, though it accentuates deficiency signs in dogs on a diet devoid of nicotinic acid,[47] whereas 3-acetylpyridine is an effective antivitamin for mice but not for bacteria [48]

5 Vitamin B_6

a. Function. Vitamin B_6 occurs naturally in the three forms, pyridoxine, pyridoxal, and pyridoxamine, which can be readily interconverted by animal tissues and microorganisms

The phosphates of pyridoxal and pyridoxamine are the functional forms of the vitamin as codecarboxylases for several enzymes concerned with decarboxylation and transamination

Pyridoxal phosphate is also the coenzyme for an enzyme in *Neurospora* that is concerned with the synthesis of tryptophan from indole and serine.

Vitamin B_6 plays an important part in fat metabolism.[49] The exact mechanism of the interrelationship is not yet clear, but the vitamin is concerned with the transport, deposition, oxidation, and synthesis of unsaturated fatty acids—particularly linoleic and arachidonic acids

The role of pyridoxine in anemia is discussed in Chapter 10

[illegible]

[illegible]

[illegible]

these compounds is inactive, thus, whereas the 4,5-diacetate was active, the 3- and 4-methyl ethers were inactive [50]

c. Antivitamins. Desoxypyridoxine (3-hydroxy-5-hydroxymethyl-2,4-dimethyl-pyridine[51] and "methoxypyridoxine" (2-methyl-3-hydroxy-4-methoxy-methyl-5-hydroxymethylpyridine)[52] antagonize pyridoxine in animals. Desoxypyridoxine is effective in most animals, whereas some species (e g , the rat and the dog) are able to demethylate "methoxypyridoxine" so that it has for them vitamin B_6 activity [49, 53]

[46] H McIlwain, *Brit J Exptl Path* **21**, 136 (1940)
[47] F M Strong, R J Madden, and C A Elvehjem, *J Biol Chem* **124**, 715 (1938).
[48] D W Woolley, *J Biol Chem* **157**, 455 (1945)
[49] [illegible]
[50] [illegible]
[51] [illegible]
[52] [illegible]
[53] [illegible]
291 (1947)

6 Pantothenic Acid

a. Function. Pantothenic acid forms a part of coenzyme A which is concerned with acetylation

b. Pantetheine.[54] **(*Lactobacillus bulgaricus* Factor).** A growth factor for *Lb bulgaricus* was isolated from natural materials.[55, 56] It was recognized as a derivative of pantothenic acid and shown to possess the structure:

$$HOCH_2\ C(Me)_2\ CH(OH)\ CO\ NHCH_2CH_2CO\ NHCH_2CH_2SH$$

Coenzyme A is probably formed by the attachment of pantetheine through a pyrophosphate linkage from the primary hydroxyl group to adenosine-2′,3′(or 3′,5′)-diphosphate[57]

c. Analogues. The naturally occurring pantothenic acid is the D-isomer, L-pantothenic acid is biologically inactive. The pantothenic acid molecule is highly specific. Many variants of the pantoic acid part of the molecule have been prepared and tested for biological activity, but they have proved inactive[58] Similarly, pantoyl derivatives of amino acids other than β-alanine have little or no activity.[59]

However, some derivatives of pantothenic acid are biologically active; thus simple esters and pantothenyl alcohol are active for rats, though not for certain microorganisms[60] (cf. p 295).

Some microorganisms can synthesize pantoic, acid and when supplied with β-alanine can satisfy their requirement of pantothenic acid.

d. Antivitamins. Pantoyltaurine, in which a sulfonyl group replaces the carboxyl group in pantothenic acid, is the best-known growth inhibitor related to it. It is an effective antagonist for the vitamin for microorganisms, but not for animals[61]

Several other pantothenic acid analogues antagonize the vitamin for microorganisms, but so far a satisfactory antagonist for animals has not been found

7 Biotin

a. Function. No coenzyme containing biotin has yet been isolated, though there is evidence that the biotin molecule is part of enzymes concerned

[54] E E Snell, G M Brown, V J Peters, J A Craig, E L Wittle, J A Moore, V M McGlohon, and O D Bird, *J Am. Chem Soc* 72, 5349 (1950)

[55] [illegible] 471 (1950).

[56] [illegible] 1950)

[57] [illegible]

[58] [illegible] McIlwain, *Biochem J* 36, 417 (1942)

[59] H. H Weinstock, E L May, A Arnold, and D Price, *J Biol Chem* 135, 343 (1940).

[60] H Pfaltz, *Z Vitaminforsch.* 13, 236 (1943)

[61] D. W. Woolley and A. G C White, *Proc Soc Exptl Biol. Med* 52, 106 (1943)

with the decarboxylation of oxalacetic acid,[62] and with the synthesis and deamination of aspartic acid [63]

Oleic acid, alone or in the presence of aspartic acid, can replace biotin for some Lactobacilli that would otherwise require this vitamin [64] This suggests that biotin functions in the synthesis of oleic acid.

b. Analogues. The biotin molecule is highly specific. Of a number of stereoisomers only the naturally occurring D-biotin is biologically active. Oxybiotin, in which an oxygen atom replaces the sulfur atom, has some biological activity for both animals and microorganisms

Some strains of *Corynebacterium diphtheriae* can use pimelic acid as a precursor for the synthesis of biotin [65]

c. Antivitamins. (1) *Desthiobiotin and biotin sulfone* In desthiobiotin, the sulfur atom of biotin is replaced by two hydrogen atoms, desthiobiotin and biotin sulfone are efficient antagonists for some bacteria,[66, 67] but they are growth-promoting substances for yeast as the latter can reconvert them to biotin [68] No compounds related to biotin have been found to antagonize the vitamin in animals

(2) *Avidin* Avidin, a protein from egg white, forms a stable compound with biotin When avidin, or raw egg white, is fed to animals, it combines with biotin in the intestinal tract, so making the vitamin unavailable to the host [69]

8 Folic Acid

a. General. The term folic acid includes several closely related compounds Folic acid occurs naturally as pteroylglutamic acid and as the tri- and heptaglutamates Pteroylglutamic acid and the di- and triglutamates have been prepared by synthesis All these forms are biologically active. Their microbiological potencies depend on the number of glutamic acid residues, thus pteroylglutamic acid is active for both *Lb casei* and *Streptococcus faecalis* R (two of the small number of microorganisms that require these factors), pteroyltriglutamic acid is active for *Lb casei* but not for *S faecalis* R, and pteroylheptaglutamic acid is microbiologically inactive.

b. Folinic Acid (Citrovorum Factor). The factor necessary for the growth of *Leuconostoc citrovorum* was shown to be closely related to folic acid [70] It is identical with synthetic material that has the structure 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid, and it has been named folinic acid [71]

[62] H C Lichstein and W W Umbreit, *J Biol Chem* 170, 329 (1947)
[63] H C Lichstein and W W Umbreit, *J Biol Chem* 170, 423 (1947)
[64] A E Axelrod, M. Mitz, and K Hofmann, *J Biol Chem* 175, 265 (1948)
[65] J [illegible]
[66] V [illegible]
[67] K [illegible] 207 (1941).
[68] K [illegible]
[69] R [illegible]

Folinic acid is believed to be the form in which folic acid is incorporat into enzyme systems

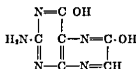
c. Function. Folic acid is required in birds and mammals for norm blood cell development (see Chapter 10). No coenzymes containing have yet been isolated, but it probably takes part in processes concern with protein utilization, particularly with the decarboxylation of tyrosin not only for hemopoiesis but also for growth and development.

Folic acid, probably in the form of folinic acid, is clearly concerned the formation and metabolism of formate and other one-carbon fragmen (see ref. 71*a*).

Stokes[72] showed that relatively large quantities of thymine or thymidi can replace folic acid for *Lb. casei* and suggested that folic acid is concern with the synthesis of these compounds.

In the chick, folic acid is involved in the conversion of choline to betai and in transmethylation from betaine to methionine.[73] Glycine toxicity young chicks can be overcome by folic acid, though more effectively l folic acid and vitamin B_{12} together [74]

d. Related Compounds. (1) *Xanthopterin.*



This yellow pigment, first isolated from the wings of butterflies,[75] *has bee* obtained from liver and prepared by synthesis.[76] It was shown to cure a anemia observed in Chinook salmon[77] and to relieve some of the signs i monkeys[78] made anemic by deficiency of folic acid. Folic acid complete cures such monkeys but is less active than xanthopterin in fish anemi which suggests that xanthopterin is active in its own right in fish but th in the monkey it is a precursor for the synthesis of folic acid Xanthopteri is ineffective in curing the anemia of folic acid-deficient chicks [79]

(2) *SLR Factor* (*Rhizopterin*)

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[70] H E Sauberlich and C A Baumann, *J Biol. Chem* **176**, 165 (1948)

[71] W Shive, T J Bardos, T J Bond, and L L Rogers, *J. Am Chem Soc* 7 2817 (1950)

[71a] A D Welch and C A Nichol, *Ann Rev Biochem* **21**, 633 (1952)

[72] [illegible]

[73] [illegible] 15 (1951)

[74] [illegible], 388 (1951)

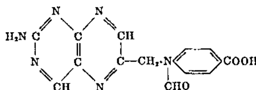
[75] [illegible]

[76] [illegible]

[77] R W Simmons and E [illegible]

[78] J R. Totter and P. L. Day, *J. Biol Chem* **147**, 257 (1943).

[79] B L O'Dell and A. G Hogan, *J Biol Chem* **149**, 323 (1943).



This compound (formylpteroic acid) was first isolated from the liquor of *Rhizopus nigricans* fermentation.[80] It is active for *S. lactis* R. but inactive for *Lb. casei*, and it can be replaced by folic acid for all bacteria that need it.[81]

e. Analogues. Many folic acid analogues have been prepared. Daniel *et al.* examined the value of about sixty compounds, mostly substituted pteridines, for growth promotion and hemoglobin formation in chicks.[82] Some of the compounds were inactive or antagonistic, whereas others stimulated growth but not hemoglobin formation, and some had the opposite effect.

Many other compounds closely related to folic acid have been prepared and some of these are of considerable interest as antivitamins.

f. Antivitamins. Pteroylaspartic acid is an efficient antagonist for microorganisms and, to a lesser degree, for the chick.[83]

Methylfolic acid (10-methylpterin) and 4-aminopteroylglutamic acid (4-aminopterin) are potent antagonists for microorganisms, mammals, and chicks.[84] 4-Aminopterin proved toxic for animals, and less toxic derivatives (e.g., 4-amino-10-methylpterin) have since been prepared.[85] These compounds are of particular interest as inhibitors of neoplastic growths.[84, 86]

9. Vitamin B_{12}

a. General. Like the vitamin B_6 group and the folic acids, the term vitamin B_{12} includes a group of closely related compounds that can be easily interconverted and that have similar physiological properties. The chemistry of this group has recently been reviewed by Smith.[87]

[80] L. L. Rickes, L. Chaiet, and J. C. Keresztesy, *J. Am. Chem. Soc.* 69, 2749 (1947).
[81] J. L. Stokes, J. C. Keresztesy, and J. W. Foster, *Science* 100, 522 (1944).
[82] L. J. Daniel, M. L. Scott, L. C. Norris, and G. F. Heuser, *J. Biol. Chem.* 173, 123 (1948).
[83] B. L. Hutchings, J. H. Mowat, J. J. Oleson, E. L. R. Stokstad, J. H. Boothe, C. W. Waller, R. B. Angier, J. Semb, and Y. SubbaRow, *J. Biol. Chem.* 170, 323 (1947).
[84] T. H. Jukes, E. L. R. Stokstad, and A. L. Franklin, *Ann. N. Y. Acad. Sci.* 52, 1336 (1950).
[85] A. L. Franklin, M. L. Belt, E. L. R. Stokstad, and T. H. Jukes, *J. Biol. Chem.* 177, 621 (1949).
[86] P. A. Little, A. Sampath, and Y. SubbaRow, *J. Lab. Clin. Med.* 33, 1144 (1948).
[87] E. L. Smith, *Nutrition Abstracts & Revs.* 20, 795 (1951).

Vitamin B_{12} itself contains a cyano group linked to the cobalt atom and has been named cyanocobalamin. Vitamins B_{12a} and B_{12b} are now known to be identical and to be hydroxocobalamin. Vitamin B_{12c}, isolated from *Streptomyces griseus* fermentation and prepared by treating vitamin $B_{12a(or\ b)}$ with nitrous acid, is nitritocobalamin A fifth postulated member of the group, vitamin B_{12d}, has now been shown to be identical with vitamin $B_{12a(or\ b)}$.[88] Vitamins $B_{12a(b)\ or\ c}$ are converted to vitamin B_{12} by treatment with sodium cyanide at pH 5, and the addition of excess cyanide at an alkaline pH causes the formation of a double cyanide which is purple in color [89]

b. Function. Vitamin B_{12} is of prime importance in red blood cell formation and hence in the prevention and cure of pernicious anemia These aspects of its function are fully discussed in Chapter 10

Like folic acid, vitamin B_{12} takes part in protein utilization, not only for hemopoiesis but also for growth and development, and also in the formation and transfer of one-carbon intermediates.[71a] From a nutritional standpoint this vitamin is of interest in its relation to the animal protein factor (APF) of which it is undoubtedly the principal, but not necessarily *the only, component* (*see p. 327*) *As young animals usually carry reserves* of vitamin B_{12} or APF factors, it is not always possible to separate the function of vitamin B_{12} itself from that of the APF, but it seems certain from experiments with rats and with chicks that vitamin B_{12} is concerned in transmethylation processes, particularly those involving methionine formation [90, 91]

Vitamin B_{12} and folic acid alone, or together, overcome glycine toxicity in young chicks,[74] these two vitamins are also concerned with the synthesis of nucleic acids [92]

c. Related Factors. Several compounds apparently closely related to vitamin B_{12} have been isolated from the rumen contents and feces of calves,[93] from pig manure,[94] from rat feces,[95] and from the culture of a rumen anaerobe [96] These compounds are active for certain microorganisms

[88] E L Smith, *Biochem J* **50**, xxxvi (1952)
[89] E Kaczka, D E Wolf, F A Kuehl, Jr , and K Folkers, *Science* **112**, 354 (1950)
[90] J A. Stekol and K Weiss, *J Biol Chem* **186**, 343 (1950)
[91] T H Jukes, E L R Stokstad, and H P Broquist, *Arch Biochem* **25**, 453 (1950)
[92] T H Jukes, H P Broquist, and E L R. Stokstad, *Arch Biochem* **26**, 157 (1950)
[93] J E Ford and J W G Porter, *Biochem J.* **51**, v (1952)
[94] ... *ite Factoren* Doc-
[95] ... *m* **194**, 539 (1952)
[96] ... . McGlohon, and ... l Society, p 22C ... (1951)

Preliminary reports that they are active for chicks and man[94–97] may require revision in view of the finding[97a] that the supposedly pure compounds were not pure but contained vitamin B_{12} and each other as impurities Their interrelationships and their relationship to vitamin B_{12} are not yet known, though Pfiffner *et al* [98] have shown that their pseudovitamins B_{12a} and B_{12b} contain adenine in place of the benzimidazole group present in vitamin B_{12}

It has been suggested that these compounds are growth factors essential for the normal metabolism of intestinal microorganisms [99]

10 Inositol

a. General. Inositol occurs in nature in the free form, as the hexaphosphoric ester (phytic acid), in some cephalins, and in certain protein complexes whose structure is not yet known

Nothing definite has been established about the function of inositol, though it may be a part of the pancreatic amylase enzyme [100]

b. Analogues and Antivitamins. The stereoisomers of *meso*-inositol, the naturally occurring form, show little or no biological activity Replacement of the hydroxyl groups by chlorine atoms gives hexachlorocyclohexane, the insecticide, which is antagonistic to inositol for yeast[101] and fungi [102]

11 *p*-Aminobenzoic Acid

a. Function. Apart from its occurrence in the molecule of folic acid, *p*-aminobenzoic acid probably functions in the synthesis of purines[103] and of methionine [104]

b. Analogues. Very few compounds related to *p*-aminobenzoic acid have growth-promoting properties, *p*-nitrobenzoic acid is active but other *para*-substituted benzoic acids are inactive,[105] esters of *p*-aminobenzoic acid are inactive or only slightly active

c. Antivitamins. The ability of *p*-aminobenzoic acid to antagonize the effect of sulfanilamide was the first and classical example of the antimet-

[97] M E Coates, J E Ford, G F Harrison, S K Kon, and J W G Porter, *Biochem J* **51**, vi (1952)

[97a] J E Ford, L S Holdsworth, S K Kon, and J W G Porter, *Nature* **171**, 148 (1953)

[98] H W Dion, D G Calkins, and J J Pfiffner, *J Am Chem Soc* **74**, 1108 (1952)

[99] J E Ford, S K Kon, and J W G Porter, *Chemistry & Industry* **1952**, 495

[100] R J Williams, F Schlenk, and M A Eppright, *J Am Chem Soc* **66**, 896 (1944)

[101] S Kirkwood and P H Phillips, *J Biol Chem* **163**, 251 (1946)

[102] H W Burton, S E Jacobs, and A Goldstein, *Nature* **158**, 22 (1946)

[103] E E Snell and H K Mitchell, *Arch Biochem* **1**, 93 (1942-1943)

[104] W Shive and E C Roberts, *J Biol Chem* **162**, 463 (1946)

[105] O H Johnson, D E Green, and R Pauli, *J Biol Chem* **153**, 37 (1944)

abolic activity of structural analogues.[106, 107] As with the other vitamin-antivitamin relationships, the effect can be reversed by the addition of excess of the factor antagonized, so that sulfonamides act as antivitamins for *p*-aminobenzoic acid, and their mode of action as antibacterial agents is believed to be by competing with *p*-aminobenzoic acid for certain enzymes essential for normal bacterial metabolism.

12. Choline

a. Function. Choline acts as a methylating agent, and as such it can be replaced by methionine or betaine. These latter substances cannot, however, carry out the other functions of choline.

The lipotropic action of choline may be due to its incorporation into phospholipin molecules,[108] which facilitate the transport and metabolism of fatty acids. Choline may also act as a stimulant to the synthesis and utilization of fats in the liver.[109]

Choline is one of the factors preventing perosis in chicks[110] and turkeys,[111] though the mechanism of its action is not known.

Finally, choline is the precursor of acetylcholine.

b. Analogues. No single compound analogous to choline can carry out all the functions of the vitamin, though several compounds can replace choline in one or more of its functions.

III. Physiological Function

1 Vitamin Requirements

a. General. The vitamins of the B complex are needed by all living cells, but, except for nicotinic acid (from tryptophan) and choline (from aminoethanol), animal tissues are incapable of their synthesis. Animals, therefore, satisfy their needs by absorbing vitamins in the alimentary tract, either from the food or from the intestinal bacteria, or, more frequently, from both It will be seen later that ruminants are, by and large, independent of an exogenous supply of B vitamins, but in other species a variety of physiological factors determines the quantity needed in the food

The term requirement as applied to the amount of a vitamin needed in the food may cover anything from an intake essential to avoid obvious signs of deficiency to that ample enough for optimal nutrition, but even if the term and the species and physiological category of the animal are

106 D. D. Woods, *Brit. J. Exptl. Path.* 21, 74 (1940)

107 (1940)

108

109 *J. Biol Chem* 176, 193 (1948)

110 T. H. Jukes, *J. Nutrition* 22, 315 (1941)

111 T. H. Jukes, *J. Nutrition* 20, 445 (1940)

rigidly defined, variations within these last two allow only fairly broad estimates cushioned by a margin of safety. (See, e g , refs 112–116) Within any one species, the normal requirements for a given vitamin are governed by four main factors the availability of the vitamin in the food, the nature of the food and the balance of nutrients, the physiological category of the animal, and the availability of the vitamin arising from intestinal synthesis.

Pathological conditions other than specific deficiencies may impose special demands or alter availability It is a truism to say that adequate nutrition is of great importance in the treatment of disease The diet must be governed by the nature of the ailment The B vitamins are no exception and their indiscriminate use is of no value in the treatment of ill-health

b. Availability of Vitamins. Most B-complex vitamins occur in nature in bound form within the cellular structure of vegetable or animal tissues. Before the vitamin can be absorbed from the food by man or animals the cellular structures must first be broken down by digestion, preceded by fermentation or cooking if need be, and the vitamin then liberated in the gut by enzyme action The process of liberation is not without hazard for some of the vitamins, since the intestinal enzymes may bring about some destruction of the vitamin itself Thus with biotin, pantothenic acid, and folic acid the peptide link may be hydrolyzed

The mechanism by which the vitamins are absorbed from the intestine has not been fully explained It is believed that thiamine[116a] and riboflavin[117] are phosphorylated in the intestinal mucosa before absorption, and a similar mechanism is probably used for the absorption of the other vitamins that can form phosphoric acid esters The absorption of vitamin B_{12} is of particular interest as it is the failure of this process that leads in man to pernicious anemia It is believed that vitamin B_{12} combines with the intrinsic factor present in normal gastric juice and that the resulting complex, which protects the vitamin from intestinal microorganisms, passes into the small intestine where the complex or the vitamin itself is taken up by the intestinal mucosa (cf ref 118). In patients suffering from pernicious anemia the gastric juice does not contain the intrinsic factor so that vitamin B_{12} in the food is not absorbed and has to be given by injection

[113] British Medical Association, *Report of the Committee on Nutrition*, Her Majesty's Stationery Office, London, 1950

[114] *Natl Research Council (U.S)*, *Reprint and Circ Ser.* 129, Washington, D C, 1948

[115] *Bull Can Council Nutrition* 2, 1 (1950)

[illegible]

[118] C C Ungley, *Nutrition Abstracts & Revs* 21, 1 (1951).

Two other factors that can reduce the availability of specific vitamins, the action of thiaminases of fish or bracken on thiamine and that of the avidin of egg white on biotin, have already been mentioned on p 300, 305 A more general factor in the economy of most vitamins is their uptake by intestinal microorganisms Certain of these absorb vitamins far beyond their own requirements and in this way actively compete with the host for the vitamins in the food. This has been strikingly demonstrated in the use of yeast as a foodstuff It has been known for some years that dried yeast is a better source of thiamine for rats than the live yeast from which it is made [119] Apparently, live yeast not only does not serve as a source of the vitamin but removes and renders unavailable some of the thiamine supplied by the rest of the diet. In this way experimental thiamine deficiency has been produced even in man.[120] It goes without saying that in this instance of bacterial competition, as in others, the organisms must pass unchanged through the absorptive parts of the gut so that even if they are broken down later on the vitamins they contain are not available to the host animal. In the same way a worm (*Diphyllobothrium latum*) living in the upper intestine can cause "tapeworm anemia" by accumulating vitamin B_{12} from the diet of the host [121]

c. Nature of Food and Balance of Nutrients. Demands for certain vitamins are clearly related to the other constituents of the diet; thus thiamine essential for the metabolism of carbohydrate is apparently not needed for that of fat, hence fat in the diet spares the vitamin [122] The amount of tryptophan in the diet and also the balance of other amino acids determine the wanted level of preformed nicotinic acid [123, 124]

Excess of one vitamin may precipitate deficiency of another. Thus Richards[125] produced signs of pyridoxin deficiency in rats on a marginal diet by giving them extra thiamine The mechanism of such imbalance is not clear, but it may possibly be connected with the saturation by one vitamin of the phosphorylating systems needed for absorption (see p. 311).

Many other interrelationships between vitamins have been reported They include that between vitamin B_{12} and pyridoxin suggested by the lowering of the vitamin B_6 content of the blood of cobalt-deficient sheep,[126]

119 [illegible] 103, 95 (1933).
120 [illegible] (1946)
121 [illegible] 266, 283 (1952)
122 [illegible] armacol Microbiol 3, 95 (1933)
123 W. A Krehl, P S Sarma, L J. Teply, and C. A. Elvehjem, *J. Nutrition* 31, 85 (1946).
124 W A Krehl, P. S Sarma, and C A Elvehjem, *J Biol Chem* 162, 403 (1946)
125 M B Richards, *Brit Med J.* I, 433 (1945)
126 S N. Ray, W C. Weir, A L Pope, and P. H. Phillips, *J. Nutrition* 34, 595 (1947)

that between folic acid and nicotinic acid in the cure of nicotinic acid deficiency (see p 325), and that between vitamin B_{12} and folic acid in anemia (see Chapter 10).

d. Requirements as Influenced by Physiological Conditions. (1) *General.* As a rule the physiological stresses of growth, reproduction, severe exercise, and exposure to exacting conditions impose additional demands for vitamins as compared with those of normal adult metabolism

(2) *Normal Adult Metabolism* There is sufficient evidence to relate the needs for thiamine and nicotinic acid in normal adult metabolism to the calorie intake[112, 113, 114] The relationship between demand and non-fat calories is also well established for thiamine[112, 114] Information about other vitamins is less definite, but it seems reasonable to relate riboflavin requirements to body weight rather than to the amount of food[113] Vitamin allowances for man are usually assessed in these ways Certain authorities recommend fixed ratios for thiamine, riboflavin and nicotinic acid With domestic animals the convention is to express their needs in terms of food and thus to relate them to calorie intake[115, 116]

(3) *Growth* In the growing animal the metabolic rate is higher than in the quiescent adult and vitamin needs are proportionately greater. The speed of growth is a determining factor, thus allowances for growing chicks and fattening pigs should be generous[115, 116] With the relatively slow-growing human infant and child these special demands are much less[112, 113, 114]

(4) *Reproductive Processes* Reproductive processes increase the demand for vitamins of the B complex not only because of increase in metabolic rate but also because of the demands of the developing fetus, and in mammals to cover secretion of these factors into milk

Eggs of birds and the colostrum and milk of mammals contain appreciable amounts of all the vitamins, though these vary considerably with the species[127, 128] As a rule, as with other constituents, the concentration of B vitamins is higher in the milk of rapidly growing species[129] The vitamin content of the milk of animals that derive most of their vitamins from their food varies with the intake When this is inadequate, the downward trend is for a time buffered by the body stores on which the mother draws. When these are exhausted, the milk may become so depleted as to induce signs of deficiency in the suckling young, e g. those of infantile beriberi in the absence of thiamine[130] and megaloblastic anemia of infancy in deficiency of folic acid[131] The constant drain throughout lactation must evidently in

[127] *Bull*
[128] S K
[129] P B
[130] D W
[131] T D

Two other factors that can reduce the availability of specific vitamins, the action of thiaminases of fish or bracken on thiamine and that of the avidin of egg white on biotin, have already been mentioned on p 300, 305 A more general factor in the economy of most vitamins is their uptake by intestinal microorganisms Certain of these absorb vitamins far beyond their own requirements and in this way actively compete with the host for the vitamins in the food. This has been strikingly demonstrated in the use of yeast as a foodstuff It has been known for some years that dried yeast is a better source of thiamine for rats than the live yeast from which it is made[119] Apparently, live yeast not only does not serve as a source of the vitamin but removes and renders unavailable some of the thiamine supplied by the rest of the diet. In this way experimental thiamine deficiency has been produced even in man[120] It goes without saying that in this instance of bacterial competition, as in others, the organisms must pass unchanged through the absorptive parts of the gut so that even if they are broken down later on the vitamins they contain are not available to the host animal In the same way a worm (*Diphyllobothrium latum*) living in the upper intestine can cause "tapeworm anemia" by accumulating vitamin B_{12} from the diet of the host[121]

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119 [illegible] 103, 95 (1933)
120 [illegible] 1946).
121 [illegible] 66, 283 (1952)
122 [illegible] *rmacol. Microbiol* 3, 95 (1933)
123 W A Krehl, P S Sarma, L J. Teply, and C. A Elvehjem, *J. Nutrition* 31, 85 (1946)
124 W A Krehl, P. S Sarma, and C. A Elvehjem, *J Biol Chem* 162, 403 (1946)
125 M B Richards, *Brit Med J.* I, 433 (1945).
126 S N Ray, W. C Weir, A L Pope, and P. H Phillips, *J Nutrition* 34, 595 (1947)

tried to single out specific vitamin formers, so far as is known now the ability to synthesize vitamins is not restricted to specialized microorganisms, and many species contribute their share, probably as a by-product of their normal metabolism

It has now been established that intestinal synthesis by microbial activity of one or more of the vitamins of the B complex takes place in many mammals and birds [139, 140] In ruminants, synthesis occurs largely in the rumen, before the food enters the true digestive and absorptive tracts, so that conditions are specially favorable for the utilization of the products In other animals the main site of microbial activity is the large intestine and, although much microbial synthesis undoubtedly occurs, the amount of vitamins available to the host is limited, possibly by the nature of the epithelium lining this part of the gut which does not appear to be adapted for the absorption of nutrients, and certainly by the fact that the synthesized material is within the microbial cells, which are not, as in the ruminant, broken down by digestive processes, but are largely excreted intact. Hence the importance of feces, when eaten, as a source of vitamins for the non-ruminant

The vitamin intake of ruminants may vary within wide limits from the large quantities in young grass to the modest supply from straw or fibrous vegetation, yet within the rumen the concentration is probably more constant Though, as will be seen later (p 317), experimental procedures may alter the rate of ruminal synthesis of vitamins, it seems likely that by some mass action a plentiful exogenous supply depresses microbial synthesis Any excess above threshold values may also be removed by direct absorption through the rumen wall and rapidly excreted Be that as it may, the vitamin economy of the ruminant is strikingly independent of outside conditions as witnessed by the constancy of the B vitamins in milk regardless of intake [141]

Omnivorous animals with relatively short guts and small caecums, such as the rat, the pig, or man, are largely dependent for their vitamins on food and get only a small and variable proportion of their needs from intestinal synthesis Herbivorous animals with longer guts and large caecums, such as the horse and the rabbit, derive more benefit from intestinal synthesis Finally, the ruminants are potentially independent of a supply in the food

An interesting example of adaptation, in which a non-ruminant can dispense entirely with an exogenous supply of vitamins, is the condition of refection in rats receiving a diet rich in raw starch Refection was first

[139] V W Najjar and R Barrett, *Vitamins and Hormones* 3, 23 (1945)
[140] S K Kon, *Proc Nutrition Soc (Engl and Scot)* 3, 217 (1945)
[141] S K Kon and K M Henry, *J Dairy Res* 16, 68 (1949).

non-ruminants be covered by increased intake, and recommended allowances will be found in publications already mentioned.

(5) *Exercise and Climate.* The effect of vigorous exercise in increasing calorie expenditure undoubtedly brings about increased metabolic demands for thiamine and possibly other vitamins of the B complex. In addition certain vitamins (folic acid, pantothenic acid, and inositol) are lost in appreciable amounts in the sweat.[132] Increased calorie expenditure in the cold has probably the same effect as exercise, but the tolerance of men to repeated exposure to cold is not increased by a more plentiful intake of vitamins of the B complex.[133]

e. Intestinal Synthesis of Vitamins. (1) *General.* In 1911 Osborne and Mendel[134] already recognized that rats subsisting on a vitamin-deficient diet frequently benefit from consuming their own droppings, and it was realized later that the beneficial effects of this practice are largely due to the content of B vitamins in the ingested feces. It was also established that these vitamins are formed by microbial synthesis in the large intestine and that they do not represent an unabsorbed excess passing unchanged through the intestinal tract (cf ref. 135).

In 1915 South African workers discovered that ruminants can exist for long periods of time on a diet very low in certain vitamins, and they wrote in a truly prophetic fashion:[136] "we think it at least possible that the vitamine requirements of cattle are so low that they may even be covered *indirectly by synthesis carried out by the extensive bacterial flora* of the intestines". This view was confirmed eleven years later when Bechdel *et al*[137] showed that heifer calves could grow to maturity and reproduce on a ration that contained insufficient "vitamin B" to support the growth of young rats for more than 2 to 4 weeks. They incidentally found that the apparent immunity of the calf to deprivation of vitamins did not extend to vitamin A, which is essential in ruminant nutrition.

Intestinal microorganisms are capable of synthesizing severally or together *almost all the vitamins of the B complex*.[138] *Although* earlier workers

[132] H Spector, T S Hamilton, and H H Mitchell, *J Biol Chem* 161, 145 (1945), R. C. Johnson, H. H. Mitchell, and T. S. Hamilton, *ibid* 161, 357 (1945)

[illegible] ahnestock, *Am J* [illegible]

[illegible] 156, Pt. 2, p 59 (1911)

[135] H K Mitchell and E R Isbell, *Univ Texas Pub* 4237, p. 125 (1942)

[136] A Theiler, H. H Green, and P R Viljoen, *Rep Vet Research S Africa*, 3 and 4, 9 (1915)

[137] S I Bechdel, C H Eckles, and L S Palmer, *J Dairy Sci*. 9, 409 (1926), S I Bechdel and H E Honeywell, *J Agr Research* 35, 283 (1927), S I Bechdel, H E Honeywell, R. A. Dutcher, and M. H Knutsen, *J Biol Chem.* 80, 231 (1928)

[138] W H Peterson and M. S Peterson, *Bact. Revs* 9, 49 (1945).

mins. Thus diets containing starch or dextrin lead to greater synthesis of vitamins in the caecums of rats than diets containing sucrose (cf. ref 140), in the ruminant the presence of readily available carbohydrate (molasses) has a stimulating effect on vitamin synthesis [151]

Sometimes the addition of a particular vitamin to the feed increases the production of other vitamins For instance, the addition of thiamine to the feed of a calf stimulated the synthesis of riboflavin, pantothenic acid, pyridoxin, and biotin [152] In this respect thiamine seems to occupy a key position and may well be a limiting factor in the microbial formation of other members of the B complex, both in the ruminant and in the refected rat [140, 141]

Recent work has shown that symbiotic vitamin synthesis may play a part in the vitamin economy of man [153, 154] In the more recent experiments[154] groups of healthy adults received diets containing subnormal amounts of one or more vitamins of the B complex Urine and feces were collected and analyzed for their vitamin content A balance sheet could then be drawn up and the input and output of a particular vitamin compared The average fecal excretion of all the vitamins studied was as high on the restricted as on the normal diet, but the average urinary output of most factors was lower on the restricted diet The combined outputs of almost all the vitamins exceeded the intakes on the restricted diet Experiments of this type prove that appreciable vitamin synthesis takes place, but the lowered urinary excretion on the restricted diet emphasizes that the value of such synthesis to the host is limited by the poor intestinal absorption of the products

(*b*) Effect of sulfonamides The insoluble sulfonamide drugs exert their bacteriostatic action by interfering with the metabolism of intestinal microorganisms, thus reducing the synthetic output of some of the vitamins; folic acid is directly affected as it contains *p*-aminobenzoic acid in its molecule (see p 298) These drugs have proved most valuable in investigations of the importance of the intestinal synthesis of vitamins in the nutrition of experimental animals [140, 155] For instance, rats fed on a synthetic diet adequate for growth become deficient in biotin and in folic acid when succinylsulfathiazole is added, the deficiency manifesting itself by loss in weight, growth being restored on addition of the two vitamins This in-

[151] M I Wegner, A N Booth, C A Elvehjem, and E B Hart, *Proc Soc Exptl Biol Med* **45**, 769 (1940)

[152] C C Lardinois, R C Mills, C A Elvehjem, and E B Hart, *J Dairy Sci* **27**, 579 (1944)

[153] V A Najjar and L E Holt, Jr, *J Am Med Assoc* **123**, 683 (1943)

[154] C W Denko, W E Grundy, J W G Porter, G H Berryman, T. E. Friedemann and J B Youmans, *Arch Biochem* **10**, 33 (1916), C W. Denko, W E. Grundy, N C Wheeler, C R Henderson, G H Berryman, T. E Friedemann, and J B Youmans, *Arch Biochem* **11**, 109 (1946)

[155] F S Daft and W H Sebrell, *Vitamins and Hormones* **1**, 49 (1943)

described in 1926 by Danish workers[142] as "a transmissible change in the intestinal content enabling rats to grow and thrive without B vitamins in the food." This and subsequent work[142, 143, 144] has shown that the occurrence of refection (most frequent with potato starch) is dependent on the presence of undigested starch and of starch-splitting organisms in the caecum of the rat. The end products of fermentation in the caecum lower the pH, and this appears to promote the growth of the microorganisms, to favor synthesis of the vitamins of the B complex, and to increase absorption The animal in fact becomes a vicarious ruminant, the caecum combining some of the functions of the rumen and of the true stomach Refection has also been produced in birds,[144a] and we would mention, just to confirm that coming events cast their shadow before them, that more than fifty years ago Eijkmann,[145] in his classical work on experimental beriberi, noticed that hens given potato starch instead of polished rice did not develop polyneuritis

In contrast to the condition of refection, in which a non-ruminant becomes independent of an exogenous supply of vitamins, ruminants normally pass during suckling through a pre-ruminant stage in which the normal *microbial population is not yet established, the rumen is undeveloped* and largely non-functional, and the young animal requires B vitamins in the diet just like man or the rat. Thus, with a synthetic milk lacking in the appropriate vitamin, signs of deficiency of biotin,[146] riboflavin,[147] thiamine,[148] or vitamin B_6[149] have been produced in the young calf, and those of riboflavin in the lamb [150]

(2) *Factors Affecting Intestinal Synthesis of Vitamins* (a) Composition of the diet The composition of the diet affects both the types and the numbers of the intestinal microorganisms It is to be expected, therefore, that changes in the diet will produce changes in the synthesis of various vita-

142 L S Fridericia, *Skand Arch Physiol* 49, 55 (1926), L S Fridericia, P. Freudenthal, S Gudjonsson, G Johansen, and N Schorebye, *J Hyg* 27, 70 (1927-28)

143 S K Kon, P M Kon, and A T R Mattick, *J Hyg* 38, 1 (1938)

144 J E Ford, K M Henry, S K Kon, J W G Porter, S Y Thompson, and F [illegible]

144a

145

146 *Biol Med* 63, 521 (1946)

147 A C Wiese, B C Johnson, H H Mitchell, and W B Nevens, *J Nutrition* 33, 263 (1947)

148 B. C Johnson, T S Hamilton, W B Nevens, and L E Boley, *J Nutrition* 35, 137 (1948)

149 B C Johnson, J A Pinkos, and K A Burke, *J Nutrition* 40, 309 (1950)

150 R W Luecke, R Culik, F Thorp, Jr, L H Blakeslee, and R H Nelson, *J. Animal Sci* 9, 420 (1950)

f. Requirements as Influenced by Pathological States other than Vitamin Deficiency. (1) *General* Thus far vitamin requirements have been discussed mainly in relation to the needs of the healthy individual under conditions of normal physiological stress These requirements may be, and often are, raised by illness or pathological conditions Such relationships are fully considered in specialist books (e g, refs 5 and 164) and reviews,[165, 166] and only a brief summary is possible here

(2) *Disorders of the Gastrointestinal Tract* Disorders of the gastrointestinal tract frequenly provoke nutritional deficiencies, including those of the vitamin B complex These may arise through malabsorption or through destruction or inactivation of nutrients Thus vomiting and diarrhea reduce the availability of all nutrients by reducing the time during which they can be absorbed Similarly, operative procedures involving short circuiting or resection of parts of the tract reduce the absorptive surfaces. Subtotal gastrectomy frequently produces achlorhydria, and this lack of acidity in the stomach may lead to destruction of thiamine and riboflavin in the food

Antacids such as alumina and magnesium trisilicate, and substances used in the treatment of diarrhea such as kaolin and fuller's earth, inactivate thiamine and probably other vitamins by adsorbing them and so rendering them unavailable for absorption

Frazer[167] has suggested that the sprue syndrome may arise as the result of microbial invasion of the stomach and upper small intestine, the microorganisms depriving the host of dietary vitamins, and so precipitating signs of deficiencies of folic acid, nicotinic acid, riboflavin, and vitamin B_6, on diets containing adequate amounts of these vitamins

The administration of antibiotics by mouth even for short periods may cause signs of vitamin deficiency in well-nourished individuals[168] The mechanism of this effect is not clear

(3) *Diseases of the Liver* Cirrhosis of the liver and other diseases that reduce hepatic function may affect coenzyme formation, as many of the vitamin → coenzyme transformations probably take place in this organ

The conditioned thiamine deficiency of chronic alcoholism (see p 323) may thus be partly due to the damage to the liver by alcoholic poisoning

(4) *Diseases of the Kidney* There is no evidence that renal disease as such increases requirements for B vitamins, but "salt-free" diets used in the treatment of nephritis may be low in vitamins, particularly riboflavin, and must be suitably supplemented Mercurial diuretics increase the excre-

[164] J Shafar, The Vitamins in Medical Practice, Staples, London, 1949
[165] B H Ershoff, *Physiol Revs* **28**, 107 (1948)
[166] G V Mann and F J Stare, *J Am Med Assoc* **142**, 409 (1950)
[167] A C Fraser, *Brit Med J* **II**, 731 (1949)
[168] Z A Leitner, *Brit Med J* **I**, 491 (1950)

dicates that the rats were dependent, to some extent, on microbial synthesis of these factors while on the unsupplemented diet. With refected rats this effect is dramatic, loss of weight, reduced excretion of vitamins, and death resulting rapidly from the administration of sulfonamides [141, 156]

(*c*) *Effect of antibiotics* *The astonishing recent discovery that pure* antibiotics promote growth when added to normal rations of poultry[157] and pigs[158] has not yet been fully explained. In chicks the effect may be twofold, first on vegetable-protein diets through a sparing action on B vitamins, and second in overcoming a growth depression caused by a hitherto undescribed transmissible "infection "[159]

A wide range of antibiotics, including penicillin, aureomycin, terramycin, bacitracin, and streptomycin, improves the growth of pigs and poultry.[160]

The variety of the chemical structures makes it unlikely that the antibiotic itself acts as a vitamin The effect is probably not systemic but local in the gut, as injected antibiotics are much less active than those given by mouth [159, 161]

Surprisingly, the antibiotic evokes little change in the numbers and types of the normal population of the gut, and it seems probable that it acts by modifying bacterial metabolism, leading either to a *decrease in* bacterial utilization of growth factors or to an increase in their synthesis, either of which would benefit the host.

(*d*) Studies with sterile (germ-free) animals. Animals acquire their gut population shortly after birth and carry it till death so that it is not easy to establish how they would do without it or what the microbes do nutritionally That they are by no means indispensable to the non-ruminant is shown by the interesting work of Reyniers and his colleagues,[162] in whose laboratory chickens and rats live, grow, and reproduce under sterile con*ditions, all contact with microorganisms being rigidly* excluded. In the chicks the results indicate little effect of the normal gut flora on the economy of the B complex vitamins on a diet richly endowed with all known B factors.[163]

156 M E Coates, K M Henry, P M Kon, S. K Kon, E H. Mawson, J E Stanier, and S Y Thompson, *Nature* 157, 262 (1946).

157 E L R Stokstad and T H Jukes, *Proc Soc Exptl. Biol Med* 73, 523 (1950)

158 H M. Edwards, T J Cunha, G B Meadows, R F Sewell, and C B Shawver, *Proc Soc Exptl Biol Med* 75, 445 (1950)

159 [illegible] G Porter, S H [illegible] 1952).

160 [illegible]

161 [illegible] *ol Med* 77, 209 (1951)

162 *Lobund Rept.* 1, 1 (1946); 2, 119 (1949)

163 J A. Reyniers, P C Trexler, R F. Ervin, M. Wagner, H A. Gordon, T. D Luckey, R. A. Brown, G J Mannering, and C J. Campbell, *J. Nutrition* 41, 31 (1950)

adequacy of the diet resulting in loss of weight and breakdown of tissues.[144, 172]

The adequacy of the vitamin supply is often assessed in man, and sometimes in animals, by measuring the urinary output of the vitamin and its metabolites, for in conditions of actual or marginal deficiency the urinary excretion is usually reduced. Such a measurement in a sample of urine taken at random during the day is not satisfactory as the level depends on many factors, including the time since a meal. A sample from a 24-hr. collection is more satisfactory, and this method is normally used with experimental animals. The range of individual variation is very great,

TABLE 2

FORMS IN WHICH B VITAMINS APPEAR IN THE URINE OF DIFFERENT SPECIES

(Vitamins identified only in the unchanged form are not listed.)

Thiamine	Thiamine
	Cocarboxylase (?)
	Pyrimidine moiety ("pyramin")
Nicotinic acid*	Nicotinic acid
	Nicotinamide
	Trigonelline
	N'-Methylnicotinamide
	N'-Methyl-6-pyridone-3-carboxylamide
	Dinicotinylornithine (chicks only)
	Quinolinic acid
Vitamin B_6	Pyridoxine
	Pyridoxal
	Pyridoxamine
	4-Pyridoxic acid

* See ref. 171 for distribution among different species.

however, and samples from a number of animals on the same diet are necessary to provide reliable data on the urinary excretion.

As 24-hr. collections of human urine are not easily obtained, vitamin status in man is usually assessed by measuring the urinary output of the vitamin before and after the administration of a test dose. This technique originally introduced for vitamin C by Harris and Ray[173] was used later in the study of thiamine deficiency.[174] In conditions of deficiency very little of the test dose is excreted, whereas well-nourished subjects excrete a relatively large proportion. The test dose method of assessing vitamin

[171] P. Ellinger and M. M. Abdel Kader, *Biochem. J.* 44, 77, 627 (1949).
[172] O. Mickelsen, D. Doeden, and A. Keys, *Federation Proc.* 4, 98 (1945).
[173] L. J. Harris and S. N. Ray, *Lancet* I, 71 (1935).
[174] L. J. Harris and P. C. Leong, *Lancet* I, 886 (1936).

tion of thiamine not only through diuresis but also by raising the concentration in the urine.

(5) *Endocrine Disorders.* There is no evidence that the needs of diabetics for B vitamins are increased.

Hyperthyroidism, by raising the metabolic rate, no doubt increases the requirement for vitamins of the B complex, but most of the evidence comes from experiments on animals

(6) *Infection* Though in malnutrition florid deficiency may be precipitated by infection, it is doubtful whether vitamins increase the resistance of well-nourished animals It is possible that changes in resistance depend on the relative requirement of the infective agent and the host for a particular vitamin If the agent's needs are higher, a vitamin deficiency might lead to increased resistance In experiments with animals thiamine deficiency improved resistance to certain virus infections[169]

So far, the only important therapeutic use of a vitamin in infection is that of *p*-aminobenzoic acid in rickettsial diseases.[164]

(7) *Shock* Shock induced by hemorrhage in dogs produces, through inactivation of cocarboxylase by breakdown to thiamine, a conditioned thiamine deficiency with characteristic increase in blood pyruvate.[170] Coenzyme I and flavin adenine dinucleotide may also be broken down

2 Vitamin Status

We have already discussed the factors influencing vitamin requirements (p 311) and have also shown (p. 314) that their relative importance depends on the architecture of the intestinal tract

With the possible exception of vitamin B_{12} (APF) the vitamins of the B complex are not stored to any appreciable extent by tissues, so that when the intake is greater than the requirement the excess is excreted in the urine The vitamins appear there not only in their unchanged state but several of them metabolized to related substances, sometimes in greater amount than the vitamin itself This fact must be borne in mind when measuring the urinary excretion of such vitamins Table 2 lists the vitamins and their metabolic products that have been identified in urine. The metabolites excreted by various species are often different, thus six metabolites of nicotinic acid are known, but most species only excrete two or three of them.[171]

It should be noted that excretion of a vitamin need not necessarily indicate that it is present in excess, as the loss may simply reflect the in-

[169] H A Waisman, H C. Lichstein, C A Elvehjem, and P. F Clark, *Arch Biochem* 8, 203 (1945)

[170] W M Govier and M. E Greig, *J Pharmacol and Exptl Therap* 79, 240 (1943)

outline and only an indication of the enormous volume of experimental work can be given Information about naturally occurring deficiency diseases in man will be found in refs 182 and 183 and a critical discussion of work on those experimentally induced in ref 183a. Lesions and signs in experimental animals are described in most textbooks on B vitamins already quoted, especially in ref. 33 Information about poultry will be found in refs. 115 and 184

b. Thiamine Deficiency. In man vitamin B_1 deficiency or beriberi is still endemic in regions where polished rice is a major item of diet It can also occur in other countries when the dietary intake of thiamine is insufficient and especially under the physiological stresses of hard work, pregnancy, lactation, or growth It can also be secondary to chronic alcoholism where the high calorie intake in the form of alcohol at the expense of other foods increases the requirement for the vitamin

Beriberi is characterized by degeneration of the peripheral nerves including a peripheral neuritis and by enlargement of the right heart, often accompanied by edema, though there is also a chronic dry form of beriberi

The edema may be extensive in severe cases, and a pulmonary edema frequently aggravates the cardiac condition leading to eventual heart failure Beriberi can usually be cured in the early stages by adequate treatment with thiamine, though, as naturally occurring beriberi is frequently accompanied by deficiencies of other vitamins of the B complex, a mixture, of vitamins is usually given Later, recovery becomes slow and there is a third stage when the pathological changes are irreversible Occasionally an acute variation occurs that brings about rapid aggravation of the symptoms and death within a few days, or that changes into the chronic irreversible illness

The nervous signs of thiamine deficiency in most animals are similar to those of beriberi in man

Thiamine-deficient rats show polyneuritis, they also develop convulsions, and they have a considerably slowed heart beat (bradycardia), which has been used as the basis of a biological assay of the vitamin.[185]

Vitamin B_1-deficient birds develop polyneuritis, but they also show the classical symptom of opisthotonos—a characteristic head retraction that is a form of convulsion and that was one of the first recorded signs of thiamine deficiency in experimental animals [186]

[182] L Nicholls, Tropical Nutrition and Dietetics, 3rd ed , Baillière, Tindall and Cox, London, 1951

[183] Y B Youmans, *J Am Med Assoc* 144, 307, 386 (1950)

[183a] S S B Gilder, *Brit Med J* I, 311 (1950)

[184] M E Coates, J E Ford, G F Harrison, S K Kon, E E Shepheard, and F W Wilby, *Brit J Nutrition* 6, 75 (1952)

[185] T W Birch and L J Harris, *Biochem J.* 28, 602 (1934)

[186] U Suzuki, T Shamimura, and S Okada, *Biochem Z* 43, 89 (1912)

status has since been used for riboflavin,[175] nicotinic acid,[176] pantothenic acid,[177] vitamin B_6,[178] and folic acid.[179]

Another technique used in the measurement of urinary excretion is that of the "fasting hour" specimen (cf. ref. 180).

Unfortunately the three methods of measuring vitamin status—24-hr. excretion, test dose, and "fasting hour" specimen—frequently give discordant results.[180, 181] Such studies must, therefore, be interpreted with caution, but, even so, they have given much valuable information about vitamin status on different diets and have been used in the calculation of vitamin requirements or recommended allowances.

3. Effects of Vitamin Deficiency

a. General. A vitamin deficiency can arise through shortcomings of the usual diet, or it can be produced experimentally by deliberately using a specially designed diet that is deficient in the factor *It is important to note* that naturally occurring vitamin deficiencies are rarely due to the lack of only one factor and that the classical human deficiency diseases, beriberi and pellagra, are frequently complicated by deficiencies of vitamins other than thiamine and nicotinic acid. Experimentally induced deficiencies can usually, though not always, be restricted to a deprivation of the factor studied

As the vitamins of the B complex are required by all living cells, a tissue that has an unusually high requirement for a particular vitamin suffers disproportionately from any deficiency of this factor and responds by specific pathological lesions.

In general, therefore, signs of avitaminosis are not necessarily those of any particular biochemical lesion, but reflect rather the diminished function of the cells of various body tissues leading to pathological conditions in the tissues concerned. These metabolic disturbances usually prevent the growth of the young and the eventual consequences of prolonged deficiency are frequently fatal in both young and old.

More specific lesions, however, are associated with deficiencies of thiamine and vitamin B_6 (see below)

The manifestations of vitamin deficiency can be presented here only in

[175] A. Keys, A F. Henschel, O Mickelsen, J. M Brozek, and J H Crawford, *J. Nutrition* **27**, 165 (1944)

[176] R A Coulson, P Ellinger, and G A Smart, *Brit Med J* I, 6 (1945), P Ellin- [illegible] *Lancet* II, 197 (1945)

[illegible] *ed* **70**, 235 (1949)

[illegible] W Boehne, *J Lab Clin. Med.* **32**, 1350 (1947)

[180] E Papageorge and G T Lewis, *J Nutrition* **34**, 301 (1947)

[181] H. H. Gifft and H M. Hanck, *J Nutrition* **31**, 635 (1946)

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[183] Y B Youmans, *J Am Med Assoc* **144**, 307, 386 (1950)

[183a] S S B Gilder, *Brit Med J* **1**, 341 (1950)

[184] M E Coates, J E Ford, G F Harrison, S K Kon, E E Shepheard, and F W Wilby, *Brit J Nutrition* **6**, 75 (1952)

[185] T W Birch and L J Harris, *Biochem J.* **28**, 602 (1934)

[186] U Suzuki, T Shamimura, and S Okada, *Biochem Z* **43**, 89 (1912)

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[176] R. A Coulson, P Ellinger, and G A Smart, *Brit Med J* I, 6 (1945), P Ellin- [illegible] *Lancet* II 107 (1945)

[177] [illegible]

[178] [illegible] *Med* 70, 235 (1949)

[179] [illegible] J W Boehne, *J Lab*

[180] E Papageorge and [illegible])

[181] H H Gifft and H M. Hanck, *J. Nutrition* **31**, 635 (1946)

the other signs, can be cured by nicotinic acid In severe deficiency the anemia cannot be so cured but responds to folic acid This suggests some metabolic connection between nicotinic acid and folic acid

Nicotinic acid deficiency in chicks is characterized by poor growth and feathering and by redness and inflammation of the mouth, sometimes perosis and dermatitis develop

e. Vitamin B_6 Deficiency. No clear cut deficiency disease in man can be attributed to the absence of vitamin B_6 from the diet, and no specific signs of deficiency were produced in one volunteer maintained for two months on a deficient diet [190] It has been suggested that vitamin B_6 deficiency may play a part in classical pellagra, particularly in the psychic disorders of the condition

The signs of vitamin B_6 deficiency in rats include poor growth, peripheral dermatitis or acrodynia, and convulsions (running fits) In chicks there is no dermatitis, appetite is poor and growth markedly depressed, the main signs are nervousness with vertigo, tremors, and often convulsions

Vitamin B_6 deficiency in mammals, though only infrequently in rats, is accompanied by a microcytic, hypochromic anemia (see Chapter 10)

A characteristic biochemical effect of the deficiency in rats is the increased urinary excretion of xanthurenic acid and decreased excretion of kynurenic acid resulting from the upset of the normal pathway of tryptophan metabolism

f. Pantothenic Acid Deficiency. Although deficiency of pantothenic acid probably occurs in man in conditions of malnutrition such as beriberi or pellagra, uncomplicated pantothenic acid deficiency has not been reported under natural conditions, nor has it been induced by a deficient diet

The deficiency syndrome in rats includes "blood-caked" whiskers due to deposition of porphyrin, greying of the hair (achromotrichia), dermatitis, adrenal damage, and soreness about the mouth and nose In severe deficiency anemia and granulocytopenia may occur, but these respond only to folic acid therapy In pigs the signs are similar and include loss of muscular coordination leading to a "goose-stepping" gait

The signs of pantothenic acid deficiency in chicks are dermatitis around the beak, retarded feathering, and lesions of the spinal cord

g. Biotin Deficiency. This deficiency does not occur naturally in man but can be induced by raw egg white (see p 305) Volunteers given a diet containing 30% of the total calories as raw egg white developed a scaly dermatitis and mental symptoms, including depression, lassitude, and panic [191]

Biotin deficiency can be produced in rats by raw egg white or by a deficient diet containing succinylsulfathiazole The signs of deficiency are

[190] W W Hawkins and J Barskey, *Science* 108, 284 (1948)

[191] V P Sydenstricker, S A Singal, A P Briggs, N M deVaughn and H Isbell, *J Am Med Assoc* 118, 1199 (1942)

Unlike some other deficiencies of vitamins of the B complex, thiamine deficiency leads to well-defined biochemical lesions of faulty metabolism of carbohydrate with increase in pyruvate and lactate in the blood. This now classic relationship was discovered by Peters.[186a]

c. Riboflavin Deficiency. Ariboflavinosis in man arises from an insufficient intake and is often associated with pellagra; it can also be produced experimentally.[187] The signs include inflammation and a purplish coloration of the tip and margin of the tongue (glossitis), lesions at the angles of the mouth (cheilosis), a scaly desquamation of the nose and ears, and vascularization of the cornea (cf. ref. 188).

The deficiency state in rats has been called "rat pellagra" and is evidenced by poor growth, rough hair, or loss of hair with dermatitis on the bald patches, corneal vascularization, and, in severe deficiency, myelin degeneration of peripheral nerves with a loss of reflexes.

Mild riboflavin deficiency in chicks causes curled toe paralysis; a more severe one leads to acute paralysis, followed by death.

d. Nicotinic Acid Deficiency. Nicotinic acid deficiency, or pellagra, is endemic in large areas of the world (mainly tropical and subtropical) and is generally associated with diets consisting mainly of maize which is very low in nicotinic acid and tryptophan (cf. ref. 189). Pellagragenic diets are frequently also low in thiamine, riboflavin, and pantothenic acid, so that pellagra is often complicated by the multiple nature of the avitaminosis which weakens the body, making it less able to withstand the nicotinic acid deficiency.

In man the three salient signs of pellagra are dermatitis, diarrhea and dementia. The dermatitis, usually bilaterally symmetrical, occurs particularly on exposed areas of the skin such as the face, backs of the hands and the front of the ankles. The intractable diarrhea of pellagra leads to the establishment of a vicious circle of vitamin deficiency by preventing proper uptake of nutrients from the diet. The changes taking place in the peripheral nervous system result in a vast range of disorders, from neurasthenia to extreme dementia and from mild disorders of sensations to polyneuritic paralysis.

Nicotinic acid deficiency can be induced in rats by a diet low in both nicotinic acid and tryptophan. Growth is suppressed, and the nicotinic acid content of the tissues is reduced.

Blacktongue is the most striking sign of nicotinic acid deficiency in dogs. The condition is characterized by dehydration and electrolyte imbalance. A macrocytic anemia *also occurs in moderate deficiency*. This anemia, and

186a [illegible] Lancet I, 1161 (1936).
187 [illegible] 53, 2282 (1938).
188 [illegible]
189 [illegible]

to assume that the list of B vitamins is closed Some natural materials, particularly liver, produce in experimental animals growth responses over and above those obtained with all known vitamins Several new factors are at present postulated, and only further research will show whether some of these are identical with, or closely related to, already characterized vitamins, and whether others are to swell the B vitamin group The more likely candidates are listed below

2 VITAMIN B_{13}

This name is given to a factor present in fish solubles and in distiller's dried solubles that stimulates the growth of rats[193] and chicks.[194]

3 GRASS JUICE FACTOR

Grass juice is claimed to contain a factor (or factors) necessary for the normal growth of rats,[195] guinea pigs,[195] and chicks [196]

4. WHEY FACTOR

Dried whey contains a factor that increases the growth of chicks[197] even when given with a diet supplying all the known vitamins and an antibiotic [198] The whey factor is probably a component of the APF (see p 308)

[193] A F Novak and S M Hauge, *J Biol Chem* **174**, 235, 647 (1948)
[194] A F Novak, S M Hauge, and C W Carrick, *Poultry Sci* **26**, 604 (1947).
[195] G O Kohler, C A Elvehjem, and E B Hart, *J Nutrition* **15**, 445 (1938)
[196] G O Kohler and W R Graham, Jr , *Poultry Sci* **31**, 284 (1952)
[197] F W Hill, *Poultry Sci* **27**, 536 (1948)
[198] J R Reed, R L Atkinson, and J R Couch, *J Nutrition* **43**, 501 (1951)

cessation of growth, "spectacle eye" caused by the loss of hair round the eyes, dermatitis on the neck, and stiffened joints.

In chicks, dermatitis on the feet and perosis are the most important signs of biotin deficiency.

h. Folic Acid Deficiency. The most important sign arising from a deficiency of folic acid in man and in experimental animals is a macrocytic anemia. The relation of folic acid to anemia is discussed in Chapter 10

Folic acid deficiency is produced in rats by a diet low in the factor and containing succinylsulfathiazole. The rats stop growing and become anemic

In chicks, a deficient diet alone is effective and the signs, besides anemia, are poor growth and feathering. The oviducts of folic acid-deficient pullets, unlike those of normal chicks, do not grow in response to the administration of stilbestrol

i. Vitamin B_{12} Deficiency. The sign of deficiency in man is pernicious anemia, discussed in Chapter 10.

Surprisingly, vitamin B_{12} deficiency is not associated with anemia in experimental animals. The deficiency is not easy to induce in them; it is usually necessary to deprive the mother of the factor so that her progeny is born, or hatched, with only small reserves. All-vegetable diets high in protein are generally used. The deficiency manifests itself by poor growth.

j. Inositol Deficiency. No signs of inositol deficiency in man have yet been reported

[illegible]

g

bald rats deficiency results in greying of the hair The sign is not specific *as it appears also in the absence of pantothenic acid;* less satisfactory evidence would indicate that deficiency of biotin, folic acid, and inositol may also lead to it.

No deficiency signs are known in man. Despite great hopes placed on it at one time, *p*-aminobenzoic acid does not prevent greying of human hair[193]

l. Choline Deficiency. Choline deficiency in man has not been reported The signs in rats are loss of weight, paralysis of the hind limbs, a hunched posture, and loss of hair. Livers show fatty infiltration, and the kidneys are hemorrhagic.

Choline is necessary for normal growth in chicks and plays a part in the prevention of perosis

IV. *Other Possible Vitamins*

1. General

Though it is probable that the vitamins already well characterized are those of greatest nutritional and therapeutic importance, there is no reason

[193] J. J. Eller and L. A. Diaz, *N. Y. State J. Med.* **43**, 1331 (1943).

similar disturbance in the myeloid series with pathologic macromyeloid cells, and a reduction in number and abnormality in type of thrombocytes [1] The diagnosis depends upon the examination of aspirated bone marrow which is usually obtained from the sternum The etiology of these anemias has led to the use of the term "megaloblastic" in reference to this class Pernicious anemia, sprue, and the megaloblastic anemias of pregnancy, infancy, and pellagra are included in the megaloblastic group

An occurrence in pregnant women in Bombay of an anemia associated with nutritional deficiency was described by Wills[2] under the term "tropical macrocytic anemia" She found that no response was obtained following the administration of purified liver extracts which were known to be effective in pernicious anemia However, the disease was alleviated by the oral administration of an extract of autolyzed yeast (Marmite) or by certain crude preparations obtained from liver Wills' articles constituted the first description of folic acid deficiency, and her work demonstrated the existence of a new factor which was effective in addition to the previously known anti-pernicious-anemia factor of concentrated liver extracts The new factor was eventually shown to be folic acid

a. Pernicious Anemia Pernicious anemia is now assumed to be due primarily to a deficiency of vitamin B_{12} caused by a degenerative defect in the gastric mucosa Normal gastric secretions contain an "intrinsic factor" which is needed for the uptake of dietary vitamin B_{12} from the digestive tract The human being who develops pernicious anemia, a disease peculiar to his species, is deprived of the intrinsic factor by a degenerative process which arrests the formation of normal gastric juice so that a conditioned deficiency of vitamin B_{12} is produced, the vitamin is present in the diet but to no avail A macrocytic anemia develops, accompanied by specific changes in the bone marrow and often followed by subacute combined degeneration of the spinal cord The physical signs include a "lemon-yellow pallor" which is due to the anemia and to the elevated icteric index caused by abnormally rapid breakdown of the atypical erythrocytes, an impaired sense of position and vibration and a positive Romberg sign, the tongue either very red with a smooth, swollen, shiny tip and lateral portions, or pale, shrunken, smooth, and shiny all over and with an attendant glossitis; and retinal pallor with flame-shaped retinal hemorrhages The laboratory findings include a mean corpuscular volume of about 97 cu μ and a color index usually greater than one, histamine-refractory gastric achlorhydria, increase of nucleated red cells including more than 2% megaloblasts in smears prepared from aspirated sternal bone marrow, leucopenia with

[1] Hal Downey (Ed), Handbook of Hematology, Paul B Hoeber, Inc, New York, 1938

[2] Lucy Wills, *Brit Med J* **1**, 1059 (1943)

CHAPTER 10

Vitamins and Hematopoiesis

T H JUKES

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I. Types of Anemia Occurring Due to Vitamin Deficiency

1 THE MEGALOBLASTIC ANEMIAS

Prominent among the results of nutritional deficiency in human subjects are certain anemias which are marked by megaloblastic erythropoiesis, a

studied by Welch and others,[10] who have noted that the administration of ascorbic acid together with folic acid causes a greater urinary excretion of citrovorum factor activity than is obtained when folic acid is administered without additional ascorbic acid The relation of this phenomenon to the megaloblastic anemia of infancy was discussed by May and co-workers [11] They concluded that 25% of the reported cases of this anemia were in scorbutic infants and that in a number of additional cases the history indicated that the ascorbic acid intake was inadequate The anemia was frequently seen in infants in the middle western United States who were reared on certain canned milk formulas However, the occurrence of the disease became much less frequent following the addition of ascorbic acid routinely to these milk formulas

d. Megaloblastic Anemia of Pregnancy. This anemia is characterized by the presence of macrocytic red blood cells, by megaloblastic changes in the bone marrow, by the presence of free hydrochloric acid in the gastric juice, and by the absence of neurological involvements The disease may occur at any age during the child-bearing period and is usually noted during the last three months of pregnancy or following therapy Most patients have a defective diet, and excessive vomiting or diarrhea is noted in about half of the cases The tongue may be sore, and a slight edema is often seen together with a pearly-white complexion The anemia tends to disappear spontaneously after the termination of pregnancy This anemia uniformly responds to folic acid and occasionally to large doses of vitamin B_{12}, although cases have been reported in which the signs and symptoms were aggravated by the administration of vitamin B_{12} and cleared up following the administration of folic acid An analogous condition in rats, "lactation leukopenia," has been described by Nelson and Evans[12] as occurring on a diet deficient in folic acid It is interesting to note that such diets do not produce folic acid deficiency in non-parturient rats unless a sulfonamide is added to the diet to depress intestinal synthesis of folic acid or unless a folic acid antagonist is administered to the animals Evidently the requirement for dietary folic acid is increased during pregnancy

II. Pernicious Anemia, Liver Extract, and the Intrinsic and Extrinsic Factors

1 Pernicious Anemia

The subject of pernicious anemia has received much attention in the medical literature for many years Minot and Strauss[13] have characterized

[10] A D Welch, *Trans Assoc Am Physicians* 63, 147 (1950)

[11] C D May, E M Nelson, C U Lowe, and R J Salmon, *Am J Diseases Children* 80, 191 (1950)

[12] M M Nelson and H M Evans, *J Nutrition* 38, 11 (1949)

[13] G R Minot and M B Strauss, *Vitamins and Hormones* 1, 269 (1943)

relative lymphocytosis and moderate decrease in platelets; and an increase in concentration of serum bilirubin which gives an indirect Van den Bergh reaction.[3] The signs are relieved by the injection of vitamin B_{12} or by the oral administration of a mixture of vitamin B_{12} with the intrinsic factor. However, the neurological degeneration may progress to an irreversible state if treatment is not started promptly.

b. Sprue. Pernicious anemia is usually described as a disease of people dwelling in temperature climates and appears to be especially common in Great Britain and the Scandinavian countries. In contrast, *sprue* is a syndrome which is more commonly encountered in the tropics. Prominent in its etiology is a periodic diarrhea in which the stools are fatty, frothy, and fetid. The patient experiences a loss in weight, weakness, and a blood picture resembling that of pernicious anemia. The fatty diarrhea and the megaloblastosis are considered diagnostic and are accompanied by the presence of free hydrochloric acid in the gastric contents following the injection of histamine. Sprue typically responds to a varying extent to the administration of folic acid.

c. Megaloblastic Anemia of Infancy. Megaloblastic anemia in infants was described by Faber,[4] who reported abnormalities in the bone marrow which occur as early as six weeks of age. The disease, under the name "goats' milk anemia" in infants, was often mentioned in German pediatric publications. Rominger[5] and György[6] described the relief of goats' milk anemia by liver and yeast, which indicated that a vitamin deficiency was involved. In the United States, a megaloblastic anemia of infancy has been described[7, 8] as being characterized by a normochromic and *usually macrocytic anemia*, a tendency towards leucopenia and neutropenia, a diminution in the platelet count which is often associated with an increased bleeding tendency, a megaloblastic bone marrow pattern, and a frequent incidence of splenomegaly. Deficiencies of folic acid and ascorbic acid have been associated with the disease, but the administration of ascorbic acid does *not produce remission In contrast, folic acid is specific*, and it has been stated by Siebenthal[9] that "megaloblastic anemia is a relatively common disease of white infants and since a complete and permanent recovery can be effected by folic acid therapy the condition should be understood by all those caring for small children "

The relation of ascorbic acid to the metabolism of folic acid has been

[3] G E Farrar, Jr , and H L Hyman, *Med Clinics N. Amer.* 29, 229 (1945)

[4] [illegible]

[5] [illegible] 89, 786 (1933)

[6] [illegible]

[7] [illegible] (1946)

[8] [illegible]

[9] [illegible]

even in the absence of hydrochloric acid It is also found in the gastric and intestinal mucosa of other species, notably pigs The concentration and identification of the intrinsic factor is a difficult task because of the cumbersome procedure involved in the biological assay

It appears that vitamin B_{12} may be regarded as the extrinsic factor although the possibility is not excluded that other substances with extrinsic-factor activity exist The intrinsic factor is assayed as follows. A patient with pernicious anemia in relapse is given a small and carefully regulated dose of vitamin B_{12}, usually 5 to 10 μg, by mouth daily for about 7 days and examinations of the blood are made daily There should be no appreciable increase in reticulocyte count during this period The administration of vitamin B_{12} is now continued, but the patient receives in addition the material which is to be tested for "intrinsic factor" activity, given simultaneously with, or within a very short time of, the vitamin B_{12} dosage A reticulocyte response then indicates that the material is an active source of the intrinsic factor

2 Liver Extract

Following the demonstration by Minot and Murphy[14] that whole liver, raw or lightly cooked, was effective in the treatment of pernicious anemia when fed at the rate of about ½ lb daily, liver extracts were manufactured for intramuscular injection in the treatment of this disease [15, 16] Raw beef liver was fractionated in 1927 [17] It was reported that no clinical activity was found in the residue from extraction of macerated liver with water The water extract was fractionated by adding it to absolute alcohol so that the final concentration of alcohol was approximately 95% by volume The precipitate, Fraction G, was found to be effective, and it was noted that Fraction G could be given by intramuscular injection by which route it was twenty-five times as effective as when given orally Numerous attempts were made to concentrate and identify the active substance in Fraction G These investigations, which were reviewed at length,[18] were uniformly unsuccessful in finding out anything about the chemical nature of the active substance, although some degree of concentration was achieved It is of much interest to note a large amount of chemical experimentation which was carried out with liver fractions which, although active clinically, were crude mixtures of many natural materials Eventually the U S Pharmaco-

[15] M Gänsslen, *Klin Wochschr* **9**, 2099 (1930)

[16] M B Strauss, F H L Taylor, and W B Castle, *J Am Med, Assoc* **97**, 313 (1931)

[17] E J Cohn, G R Minot, G A Alles, and W T Salter, *J Biol. Chem* **77**, 325 (1928)

[18] Y SubbaRow, A B Hastings, and M Elkin, *Vitamins and Hormones* **3**, 238 (1945)

pernicious anemia as a condition occurring particularly in the fourth and fifth decades of life and rarely before the age of 30, apt to develop in blue-eyed individuals of the Nordic race There may be a tendency towards familial occurrence. The disease is characterized by a smooth, shiny, atrophic tongue, gastric achlorhydria, and neural manifestations which may be limited to parathesias of the hands and feet or may include severe damage to the posterior and lateral columns of the spinal cord, usually marked by a diminution of vibratory sensations. *The neural manifestations may be present long before there is clear-cut evidence of macrocytic anemia*

The early history of the successful treatment of pernicious anemia with whole liver and with liver extract has been frequently reviewed It was found by Minot and Murphy[14] and their associates that the oral administration of 120 to 240 or more grams of cooked calves liver and beef liver, together with a generous diet, brought about a remission of pernicious anemia together with marked symptomatic improvements except for pronounced disorders due to spinal cord degeneration. It seems in retrospect that the effect of liver upon these first patients was most probably due to its content of folic acid In contrast, the effects which were produced in subsequent patients by the injection of liver extract may be presumed to have been largely due to vitamin B_{12}. These conclusions are supported by the observations on the extrinsic and intrinsic factors carried out by Castle and his associates These studies demonstrated that the oral administration of normal gastric juice together with dietary materials typified by lean beef produced a response in pernicious anemia, although neither gastric juice nor lean beef was effective by itself This showed that two substances were involved in producing remissions, the "extrinsic factor" which is present in foods such as beef muscle, milk, and liver, and the "intrinsic factor" which is present in normal gastric juice.

When beef was fed to a normal subject and was then recovered from his stomach after exposure to the action of gastric juice, the resulting material had therapeutic potency when fed to patients with pernicious anemia No potency was found in beef which had been recovered from the stomach of a patient with pernicious anemia These and similar experiments which were repeated and confirmed serve to show that pernicious anemia is a deficiency disease which is due to the lack of a substance which is present in normal gastric secretions but which is not formed by the stomach of a patient suffering from the disease This substance, the intrinsic factor, functions by enabling the extrinsic factor, now presumed to be vitamin B_{12}, to be taken up from the gut

The intrinsic factor is labile to heat and is found always in human gastric juice if free hydrochloric acid is secreted, however, the factor may be present

[14] G. R. Minot and W. P Murphy, *J Am Med Assoc* 87, 470 (1926)

3. Intrinsic Factor

The intrinsic factor is said to be always present in gastric juice if free hydrochloric acid is secreted, however, the factor may be present even if hydrochloric acid is absent The activity of the factor is destroyed by boiling for 5 min. or by heating at 70° to 80° for 30 min It will pass through a Berkefeld No 5 filter, it is non-dialyzable and is not identical with pepsin, rennin, and trypsin [24] The factor is present in the normal gastric juice of man and various other mammals It is present in mucosa of the small intestine of pigs It can be studied only by measuring its effects on patients with pernicious anemia in relapse, although a new test has recently been proposed by Welch and Heinle [25] These workers have suggested that the administration of intrinsic factor to patients with pernicious anemia in remission may increase the uptake of vitamin B_{12} from the intestine and that by the oral administration of vitamin B_{12} tagged with radioactive cobalt it may be possible to measure the effect of intrinsic factor in a patient in remission

A number of proposals have been made with regard to the manner or mode of action of the intrinsic factor which have not been generally accepted

(a) It was suggested by Wilkinson and co-workers[26] that the intrinsic factor combined with the extrinsic factor *in vitro* to form a new thermostable compound which was effective against pernicious anemia when given by mouth However, later investigations by various workers led to the conclusions that no such thermostable compound was formed and that the oral effectiveness of mixtures of the extrinsic and intrinsic factors was destroyed by boiling The simultaneous presence of the intrinsic and extrinsic factors in the digestive tract appears to be necessary for the uptake of the extrinsic factor from the gut

(b) It was postulated by Ågren and Waldenstrom[27] that the intrinsic factor was an aminopolypeptidase They reached this conclusion on the basis of experiments designed to concentrate aminopolypeptidase from extracts of hog stomach This viewpoint is not shared by Schilling and co-workers,[28] who found that thymus aminopolypeptidase had no intrinsic factor activity and also that normal human gastric juice did not contain aminopolypeptidase The conclusion was reached that the concentration of the two factors was coincidental in the procedure which had been used by Ågren and Waldenstrom [27]

[24] W B Castle, *Am J Med Sci* **178**, 748 (1929)
[25] A D Welch and R W Heinle, *Pharmacol Revs* **3**, 345 (1951)
[26] L Klein and J F Wilkinson, *Biochem J*. **28**, 1684 (1934)
[27] G Ågren and J Waldenström, *Acta Med Scand* **196**, 432 (1947)
[28] R F Schilling, J. S Fruton, B H J Hofstee, A D Welch, J W Harris, F H. Gordner, and W B Castle, *J Lab Clin Med* **36**, 942 (1950)

poeia placed a "ceiling" on the potency of liver extracts at 15 U.S P. units per milliliter

Many years elapsed before the active substance in liver extract was isolated, and the slow rate at which this work proceeded was due in large part to the fact that the only test subject was the patient with pernicious anemia in relapse Eventually it was noted by E Lester Smith[19] that a reddish color was correlated with anti-pernicious anemia activity in liver extracts, and he found it possible by means of partition chromatography on silicic acid columns to separate a red material which was active against pernicious anemia and also against subacute combined degeneration of the spinal cord At about the same time Rickes and co-workers[20] isolated from liver by an undescribed procedure a crystalline compound which in microgram quantities produced positive hematological responses in initial tests in patients with Addisonian pernicious anemia These workers used *L lactis* Dorner in some of their investigations and found that this microorganism responded to crystalline vitamin B_{12} Within a short time, the isolation of vitamin B_{12} from liver extract was also made by Petrow and co-workers,[21] who described its absorption spectrum.

The relation between vitamin B_{12} and the extrinsic factor was studied by Berk and co-workers [22] They reported that the administration of 5 to 10 μg of vitamin B_{12} daily by mouth to a patient with pernicious anemia in relapse produced no response. However, when gastric juice was administered simultaneously with the same doses of vitamin B_{12}, a definite response occurred. When each of the two materials was administered daily but with dosage separated by an interval of 12 hr., no response was produced These findings indicated that a substance which behaves similarly to the intrinsic factor is present in normal gastric juice and is involved in the utilization of orally administered vitamin B_{12} It followed that the anti-pernicious-anemia activity of concentrated liver extract could be attributed to the same substance which had the properties of the extrinsic factor, namely, vitamin B_{12} Similar observations were reported by Hall and co-workers[23] in 1949 who found that normal human gastric juice after sterile filtration had the property of producing a remission in pernicious anemia when given orally together with vitamin B_{12} The latter substance was ineffective in the doses used when given by mouth without gastric juice.

19 E Lester Smith, *Nature* **161**, 638 (1948)

20 E L Rickes, N G Brink, F. R Koniuszy, T R. Wood, and K Folkers, *Science* **107**, 396 (1948)

21 B. Ellis, V. Petrow, and G F Snook, *J. Pharm Pharmacol.* **1**, 60 (1949)

22 L. Berk, W B. Castle, A D Welch, R W Heinle, R M Anker, and M Epstein, *New Engl. J. Med* **239**, 911 (1948)

23 B E Hall, E H Morgan, and D C Campbell, *Proc Staff Meetings Mayo Clinic* **24**, 99 (1949).

(e) Glass and co-workers[34] suggested a relationship between glandular mucoprotein from gastric juice and the intrinsic factor of Castle They state that it appears permissible to consider the glandular mucoprotein fraction of gastric "dissolved mucin" as the main carrier of the intrinsic factor activity of human gastric juice This conclusion was based upon the protein-like properties of both substances together with certain similarities in their chemical behavior including a resistance to acid peptic digestion However, with a dose of 10 to 20 μg of vitamin B_{12}, 100 mg of gastric mucroprotein per day was usually insufficient, and the response was obtained only when this amount of B_{12} was administered daily with a dose of 150 to 200 mg of mucoprotein Welch and Heinle[35] have described responses to fractions from gastric juice when administered in quantities as low as 0 6 mg daily, so it would appear that concentrates of intrinsic factor can be prepared which are far more potent than gastric mucoprotein

(f) Callender and Lajtha[35] have postulated that an "extra-gastric" intrinsic factor is concerned in the utilization of vitamin B_{12} both in normal subjects and in patients with pernicious anemia This conclusion is based on the following observations (1) Vitamin B_{12} is inactive *in vitro* in maturing cultures of megaloblasts unless a preparation of the gastric intrinsic factor is simultaneously added (2) However, vitamin B_{12} is active when injected in pernicious anemia patients without the addition of the gastric intrinsic factor This leads to the postulation of the existence of an extra-gastric intrinsic factor present both in normal subjects and in patients with pernicious anemia which forms the "hemopoietic factor" by combination with vitamin B_{12} The gastric intrinsic factor must also be assumed to form the "hemopoietic factor" with vitamin B_{12} to account for the *in vitro* observations The "hemopoietic factor" may be a non-dialyzable thermolabile vitamin B_{12}-protein combination, and some evidence is found for this in an observation that the microbiological potency of blood serum increases following heating [36]

It was found by Horrigan and co workers[37] that injection of a single small dose of vitamin B_{12} into the bone marrow cavity of the iliac crest of patients with pernicious anemia in relapse caused local maturation within a few hours of the megaloblasts in a sample of bone marrow which was aspirated from the vicinity of the injection However, megaloblasts which were aspirated at the same time from the contralateral iliac crest showed no evidence of maturation In contrast to the activity of vitamin B_{12}, neither folic acid nor citrovorum factor was active in experiments of this type.

[34] G B J Glass, L J Boyd, M A Rubinstein, and C S Svigals, *Science* 115, 101 (1952)
[35] S T E Callender and L G Lajtha, *Blood* 6, 1234 (1951)
[36] G. I M Ross, *Nature* 166, 270 (1950)
[37] D L Horrigan, T. Jarrold, and R W Vilter, *J Clin Invest* 30, 31 (1951)

(c) A suggestion to explain the action of the extrinsic factor was made by Ternberg and Eakin.[29] They noted that a vitamin B_{12}-binding effect could be measured by microbiological assay of certain materials with *Escherichia coli* on a culture medium containing sulfanilamide at a level sufficient to inhibit growth of the organism in the absence of viamin B_{12}. This inhibitory effect was reversed by vitamin B_{12}, but the effect of the vitamin was abolished by the simultaneous addition of unheated protein fractions from gastric juice or by extracts of mucosa of the stomach or of the small intestine of the pig. It was concluded that these fractions contained a non-dialyzable heat-labile substance termed "apoerythein" which formed a complex with vitamin B_{12}, thus rending it unavailable to *E. coli*. The complex formed between vitamin B_{12} and apoerythein decomposed upon heating at 120° for 15 min., liberating the vitamin. The authors concluded that their experiments pointed to the probability that apoerythein was the intrinsic factor of Castle or an important component thereof, but no clinical investigations to test this point were described. However, a lack of specificity for the "binding" effect was described by Prusoff and co-workers,[30] who found that fractionation of solutions obtained from hog gastric mucosa resulted in the major part of the intrinsic factor activity becoming concentrated in a fraction which showed less vitamin B_{12}-binding activity than certain other fractions as measured microbiologically. Welch and Heinle[25] have noted the possibility that intrinsic factor when obtained in a state of chemical purity will prove capable of forming a complex with vitamin B_{12} which may be responsible for the physiological action of the factor but that the lack of specificity of the B_{12}-binding effect measured microbiologically indicates that this procedure is not of value as a test for the intrinsic factor.

(d) A suggestion was made by Meyer and co-workers[31] that lysozyme had intrinsic factor activity based on observations with concentrates prepared from egg white. However, more recently, work by Hall[32] has shown that the addition of lysozyme to vitamin B_{12} did not enhance the oral activity of the vitamin when administered to patients with pernicious anemia in relapse. Bird and Hoevet[33] found that concentrates of intrinsic factor bind vitamin B_{12} but not thymidine in the assay with *Lactobacillus leichmannii* and that vitamin B_{12} was dialyzable from a mixture with lysozyme but not from the complex formed by combination of the vitamin with the preparation containing the intrinsic factor.

29 [illegible] 2858 (1949)

30 [illegible]

31 [illegible] 205 (1950)

32 B. E. Hall, *Brit. Med. J.* ii, 585 (1950)

33 O. D. Bird and B. Hoevet, *J. Biol. Chem.* 190, 181 (1951).

III. Folic Acid in Nutrition and Hematopoiesis

1 Folic Acid Deficiency in Experimental Animals

Studies with various animals which were kept on purified or otherwise restricted diets showed that anemia and leukopenia often developed and could be relieved by crude supplements obtained from liver and yeast The observations of Wills and Evans[45] showed that an anemia could be produced in monkeys by feeding a restricted diet and that this anemia responded to a substance present in liver and yeast which was not precipitated by treatment with saturated ammonium sulfate This precipitation technique was shown to remove the anti-pernicious anemia factor from liver extract The factor active against anemia in monkeys was also differentiated from thiamine, riboflavin, and nicotinic acid

It was found by Day and co-workers[46] that anemia and leukopenia were produced in monkeys which were fed a diet of cooked polished rice, brown wheat, and purified casein, supplemented with cod-liver oil, salt mixture, and orange This "cytopenia" was accompanied by ulceration of the gums and diarrhea The term "vitamin M" was proposed for an unknown factor in yeast and liver which prevented the deficiency disease Eventually it was shown that pteroylglutamic acid had the nutritional properties of vitamin M

Other investigators[47] also studied a nutritional leukopenia in monkeys which received a purified diet including thiamine, riboflavin, pyridoxine, niacinamide, calcium pantothenate, and ascorbic acid It was later shown that small doses of pteroylglutamic acid relieved the deficiency and produced hematological improvement in such monkeys

A macrocytic hyperchromic anemia was produced in chicks in experiments carried out by Hogan and Parrott[48] A factor preventing the anemia was found to be adsorbed on fullers' earth at pH 1 and was given the name "vitamin B_c" The vitamin was evidently similar to "factor U" which had been described by Stokstad and Manning[49] as being needed by chicks on a diet of rice and fishmeal supplemented with the known vitamins Stokstad, using *Lactobacillus casei* as a test organism, eventually crystallized the factor,[50] which was identified as pteroylglutamic acid The role of the new vitamin in hematopoiesis in the chick was studied by Campbell, Brown, and

[45] Lucy Wills, B D F Evans, and P W Clutterbuck, *Lancet* **232**, 311 (1937); Lucy Wills and B D F Evans, *Lancet* **235**, 416 (1938)

[46] P L Day, W C Langston, and C F Shukers, *J Nutrition* **9**, 637 (1935)

[47] H E Wilson, C A Doan S Saslow, and J L Schwab, *Proc Soc Exptl Biol Med* **50**, 341 (1942)

[48] A G Hogan and E M Parrott, *J Biol Chem* **132**, 507 (1940)

[49] E L R Stokstad and P D V Manning, *J Biol Chem* **125**, 687 (1938)

[50] E L R Stokstad, *J Biol Chem* **149**, 573 (1943)

These findings appeared to indicate that vitamin B_{12}, rather than the folic acid or citrovorum factor, was the substance which was active in causing maturation of megaloblasts. This conclusion is in direct contrast to the postulations of Callender and Lajtha.[33] It has been suggested by Welch and Heinle[25] that folic acid and citrovorum factor may diffuse rapidly from the site of administration in the marrow while vitamin B_{12} may be bound by the proteins in the locality of the injection. However, there is no evidence for the latter suggestion; the intramuscular injection of 1 μg of vitamin B_{12} daily has been repeatedly observed to produce a systemic effect in pernicious anemia in relapse, thus indicating that even so small an amount of vitamin B_{12} is not "trapped" locally in the vicinity of the site of injection.

4. Extrinsic Factor

By definition, the extrinsic factor is a vitamin-like substance present in foods, the activity of which is enhanced in pernicious anemia by the oral administration of normal gastric juice As noted above, vitamin B_{12} has the properties of the extrinsic factor, and similar properties have been noted for vitamin B_{12b} [38] However, the possibility that substances not belonging to the vitamin B_{12} group of compounds may have extrinsic factor activity has been suggested It was noted by Strauss and Castle,[39] by Ungley,[40, 41] and by Wintrobe[42] that the oral activity of certain yeast fractions was increased by the simultaneous administration of gastric juice The absence of vitamin B_{12} in yeast has been consistently noted in experiments with deficient animals, and microbiological assays do not indicate its presence The presence of folic acid in yeast undoubtedly contributes to its clinical activity The earlier findings should be repeated with modern experimental criteria before the presence of an "extrinsic factor" in yeast can be accepted *Comparatively large doses, 3 to 5 mg. of* vitamin B_{12}, by mouth have been found to produce substantial and prolonged hemopoietic remissions in pernicious anemia without the addition of intrinsic factor,[43, 44] from which it may be concluded that small amounts of vitamin B_{12} can enter the blood stream from the gut if the oral dosage is sufficiently large

[38] H Lichtman, J Watson, V Ginsberg, J. V Pierce, E L R Stokstad, and T H. Jukes, *Proc Soc Exptl Biol Med* **72**, 643 (1949)
[39] M B. Strauss and W B Castle, *New Engl J Med* **226**, 1013 (1942)
[40] C C Ungley, *Quart J Med* **2**, 281 (1933)
[41] C C Ungley and G V James, *Quart J Med* **3**, 523 (1934)
[42] M M Wintrobe, *Am J Med Sci* **197**, 286 (1939)
[43] C C Ungley, *Brit Med J* **11**, 905 (1950)
[44] B F Chow, *J Nutrition* **43**, 323 (1951)

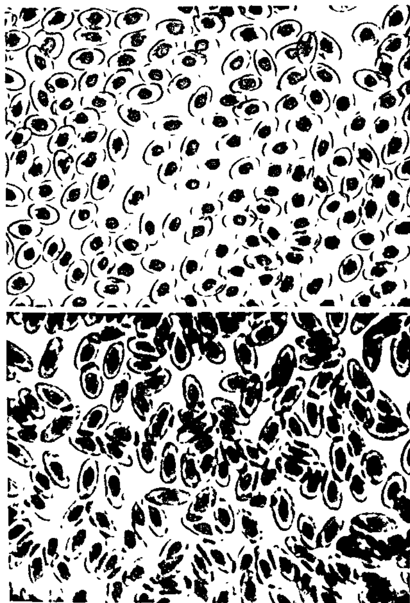


FIG 1 (*A*) Normal appearing erythrocytes from a young turkey receiving a purified diet supplemented with 1 mg pteroylglutamic acid per kilo (×1500) (*B*) Abnormal erythrocytes from a corresponding animal receiving the diet with pteroylglutamic acid omitted (×1500)

Emmett.[51] They found that 0.4 part of pteroylglutamic acid per million of diet was sufficient for normal hemoglobin, hematocrit, red cell count, and thrombocyte values, but a level of about four parts per million was required for the production of normal leucocyte levels. A comparison of injected and oral dosages indicated that subcutaneous injection was slightly more effective than feeding by pipette although differences in the two routes of administration were not marked.[52]

The occurrence of a blood dyscrasia in turkeys characterized by macrocytosis and elongation of the erythrocytes was described by Jukes and coworkers.[53] The findings are illustrated in Fig. 1.

A deficiency of folic acid may be produced in rats by adding a sulfonamide to a purified diet from which folic acid has been omitted. It appears probable that the dietary need of the rat for folic acid is satisfied by the production of this factor by the intestinal bacteria on purified diets, and that the addition of sulfonamides to such diets depresses the growth of these bacteria and results in the appearance of a deficiency syndrome which is characterized by agranulocytosis, leukopenia, anemia, and slow growth. Administration of any of several substances with folic acid activity will prevent or cure this deficiency syndrome which may also be produced by administering a folic acid antagonist, as described in Section IV. Under the physiological stress of lactation, folic acid deficiency has been found to occur in rats even when a sulfonamide was not added to the diet.

Kornberg and co-workers[54] found that a dyscrasia due to folic acid deficiency in rats could be produced by feeding purified diets which were low in pantothenic acid. Under these conditions anemia, leukopenia, and granulocytopenia were prevented by adding pantothenic acid, and it was concluded that the administration of pantothenic acid appeared to prevent the development of folic acid deficiency. Severe granulocytopenia and anemia were produced in rats fed protein-free diets. The condition was prevented by casein and not by pteroylglutamic acid; however, casein did not correct the granulocytopenia once it had developed unless folic acid was also given. A macrocytic anemia with characteristic hyperplastic changes in the bone marrow is developed by pigs under conditions of folic acid deficiency which are induced by administering an antagonist. The association of anemia with folic acid deficiency in so many different species of [illegible]

[51] [illegible] D. A. Brown and A. D. Emmett, J. Biol. Chem. 152, 483 (1944)
[52] [illegible] 164, 721 (1944)
[53] [illegible], 1 (1947)
[54] A. Kornberg, [illegible] 31 (1945)

B_{12}, but in some instances improvement was recorded only after pteroylglutamic acid had been administered The relation of ascorbic acid deficiency to the metabolism of folic acid and to the development of anemia is discussed in Section VI

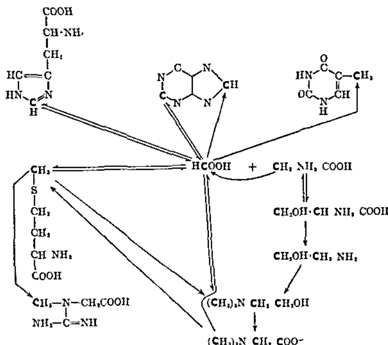


Fig 2 Metabolic transformations involving the "single-carbon" factor.

3 Biochemical Functions of Folic Acid

Folic acid has been shown to be concerned in various biological systems with the incorporation of the "single-carbon unit" into the 2 and 8 positions of the purine ring and the 5-methyl group of thymine Folic acid is also concerned in the reversible formation of serine from glycine and "formate," and there are experiments which relate folic acid to other reactions involving the single carbon unit and including the formation of choline, creatine, and histidine These relationships are diagrammatically illustrated in Fig 2.

One of the earliest observations relating folic acid to such reactions was the finding that thymine, a constituent of the desoxyribonucleic acids, was an essential nutrition factor for *Streptococcus faecalis* R. Purines, in addition to thymine, are required to replace folic acid in the nutrition of both *S.*

2. Folic Acid in the Clinical Treatment of Anemias in Human Subjects

A characteristic hemopoietic response is obtained when folic acid is administered to patients with pernicious anemia, sprue, or other megaloblastic anemias The usual dosage is 5 to 10 mg. daily, orally or by injection, although smaller amounts have often been found to be sufficient.

The megaloblastic anemia of pregnancy, described on p 331, is one of the most characteristic conditions of folic acid deficiency in human subjects Many early observations, following the early report by Wills,[2] served to differentiate the curative agent for this disease from the anti-pernicious-anemia factor of liver extracts. These earlier investigations were reviewed by Jukes and Stokstad.[55] In 1945 the use of pteroylglutamic acid in a case of megaloblastic anemia of pregnancy was described by Moore and co-workers [56] The patient had a red blood cell count of about 1.1 million red cells per cubic millimeter 18 days postpartum. She responded promptly to an intramuscular dose of 20 mg. for 10 days. Similar results were described by Spies[57] in three patients who were treated with 20 to 50 mg of pteroylglutamic acid daily Three cases which had failed to respond to the injection of liver extract were successfully treated with pteroylglutamic acid by Davidson and co-workers [58]

The etiology of the megaloblastic anemia of infancy was described on p 330. The relation of folic acid to this anemia was first studied by Zuelzer and co-workers.[7, 8] They described the treatment of twelve patients with 5 to 20 mg. of pteroylglutamic acid per day for 8 days to 3 weeks Three of the children died with severe infections, and the remaining nine responded, showing reticulocyte peaks, a return of the bone marrow pattern to normal, and increases in red cell counts and hemoglobin. No further response was obtained when ascorbic acid was administered following treatment with folic acid, and no relapses were observed up to 10 months This anemia was found to develop commonly in children who had received a diet made from diluted dried cows' milk, and the disease was often associated with scurvy. Similar observations were reported by Luhby and Wheeler,[59] who found that no response was obtained to crystalline vitamin B_{12} Other investigators[60, 61, 62] reported occasional responses to vitamin

[55] T H Jukes and E L R Stokstad, *Phys Rev* 28, 51 (1948)

[56] C V. Moore, O S Bierbaum, A D Welch, and L D Wright, *J Lab Clin Med* 30, 1056 (1945)

[57] [illegible] *J Am Med Assoc* 130, 474 (1946)

[58] [illegible] , 819 (1948)

[59] [illegible] 3, 21 (1949)

[60] [illegible] 29 (1949)

[61] [illegible] *Pediatrics* 4, 723 (1950)

[62] P Sturges and G Carpenter, *Blood* 5, 458 (1950)

series of experiments by Bennett and co-workers,[71] who found that a crude liver extract ("3 3 units per cc ") enabled rats to grow on a methionine-deficient diet containing homocystine whereas no growth was obtained when a more refined liver extract ("15 units per cc ") was administered The latter liver extract was found to be extremely low in folic acid,[72] and later it was noted that both vitamin B_{12} and folic acid were needed to produce growth in rats under the conditions described by Bennett [73] The growth-promoting effect of the crude liver extract was therefore due to the fact that it contained both folic acid and vitamin B_{12} while the purified liver extract contained vitamin B_{12} alone Choline and betaine are linked with these findings, for, as originally shown by du Vigneaud *et al*,[74] in rats on a methionine-free diet with added homocystine growth takes place if choline or betaine is added to their dietary regimen even though sources of vitamin B_{12} and folic acid are not supplied It therefore appears that the utilization of certain precursors (? serine) of the methyl group of methionine is diminished by a deficiency of folic acid in the diet of rats and that these precursors may be replaced by choline even though folic acid is not supplied Succinylsulfathiazole was added to the diets used by Bennett,[73] this depressed the intestinal formation of folic acid and enabled the experimental effects described above to take place

Similar relationships between folic acid and "methylating compounds" have been observed in chicks Schaefer and co-workers[75] noted an interrelationship between the folic acid, vitamin B_{12}, and choline requirement of chicks The folic acid requirement appeared to be somewhat less when the diet was supplemented adequately with choline and vitamin B_{12} than when these supplements were omitted Experiments in our laboratory have indicated an interrelationship between folic acid and the formation of homocystine from methionine in chicks These results are shown in Table 1.

It was found by Drysdale and co-workers[76] that rats deficient in folic acid incorporated less of a standard dose of C^{14}-formate into the purines of their liver nucleic acids than did rats which had received folic acid No corresponding reductions were noted in the C^{14} counts of the visceral purines of the deficient rats Degradation of adenine and guanine showed that the C^{14} was distributed approximately equally between the 2- and 8-carbon atoms

Recent investigations have shown that a biological derivative of folic

[71] M A Bennett and G Toennies, *J Biol Chem* 163, 235 (1946)
[72] E L R Stokstad and T H Jukes, *Proc Soc Exptl Biol Med* 62, 112 (1946)
[73] M A Bennett, *Science* 110, 589 (1949)
[74] V du Vigneaud, J P Chandler, A W Moyer, and D M Keppel, *J Biol Chem.* 131, 57 (1939)
[75] A E Schaefer, W D Salmon, D R Strength, and D H Copeland, *J. Nutrition* 40, 95 (1950)
[76] G R Drysdale, G W E Plaut, and H. A Lardy, *J. Biol. Chem* 193, 533 (1951)

faecalis R and *L. casei* in a medium containing amino acids.[63, 64] Thymine could not be replaced by uracil or cytosine, but the purine requirements could be met by guanine, xanthine, adenine, or hypoxanthine. Later investigations showed that these findings could be interpreted by the functions of folic acid in catalyzing the addition of the methyl group to the pyrimidine ring to form thymine and in transferring the single carbon unit to the 2 and 8 positions in the biological formation of the purine ring Studies by Rogers and Shive[65] indicated that the "inhibition index" obtained with *x*-methyl folic acid in *L. casei* was 30 in the absence of purines and was increased to 100 by supplements of purines and to 1000 in the presence of the purines and thymine. With *S. faecalis* R , Stokstad and co-workers[66] found that *x*-methyl folic acid inhibited the growth of *S. faecalis* R. in the presence of folic acid, folic acid plus adenine, or folic acid plus thymine However, when both thymine and adenine were added to the culture medium, growth of the organism took place and the antagonist had no inhibitory effect.

Relationships between glycine, formate, and serine were investigated by Sakami[67] and others[68] with the use of radioactive carbon. It was found that glycine could give rise to formate which could combine with a second molecule of glycine to form serine. The carbon of formate or the α-carbon atom of glycine could serve as a source of methyl carbon of choline or methionine Folic acid has been shown to be involved in the synthesis of serine in bacteria,[69] in the conversion of serine to glycine in rats, and in the transfer of formate carbon into serine and other amino acids in rats.[70] Animals which were deficient in folic acid were shown to have a diminished rate of utilization of isotopically tagged compounds involved in these reactions, and the rate was restored to normal by the administration of folic acid.

The methyl groups of the choline molecule may arise from "formate," and the side chain of the molecule can be shown to be formed from aminoethanol, which is in turn produced by the decarboxylation of serine Thus all the carbon atoms in choline may become "tagged" biologically following the administration of radioactive formate to a rat The effect of folic acid on the formation of methionine in rats became apparent as a result of a

[63] E E Snell and H K Mitchell, *Proc Natl Acad Sci. U. S* **27**, 1 (1941).
[64] E L R. Stokstad, *J. Biol Chem* **139**, 475 (1941)
[65] L L Rogers and W Shive, *J Biol Chem* **173**, 751 (1948)
[66] E L R. Stokstad, J V Pierce, T. H. Jukes, and A L Franklin, *Federation Proc* **7**, 193 (1948).
[67] W Sakami, *J. Biol. Chem.* **176**, 195 (1948)
[68] T Winnick, I. Moring-Claesson, and D M Greenberg, *J Biol Chem* **175**, 127 (1948)
[69] B R Holland and W W Meinke, *J Biol Chem* **178**, 7 (1949)
[70] G W. E Plaut, J. J Betheil, and H A Lardy, *J. Biol Chem* **184**, 795 (1950)

sible to devise methods for synthesizing analogous compounds in a search for biological antagonists of folic acid.

The first preparation of this type to be described was prepared by condensing 2,4,5-triamino-6-hydroxypyrimidine with glutamic acid, but instead of dibrompropionaldehyde, which is used in the synthesis of folic acid, dibrombutyraldehyde was used so as to introduce an additional methyl group into the product. Martin and co-workers[80] used D-glutamic acid in their synthesis and termed the resultant product "7-methyl folic acid," whereas Hultquist and Smith[81] used L-glutamic acid and termed the resulting preparation "crude x-methyl pteroylglutamic acid."

TABLE 2

TOTAL FOLIC ACID ACTIVITY OF VARIOUS TISSUES, ASSAYED WITH *S. faecalis* R AND CITROVORUM FACTOR ACTIVITY, ASSAYED WITH *Le. citrovorum* 8081[77, 78]

Material	Folic acid (total)	Citrovorum factor
	μg./g.	μg./g.
Liver, rat	1.72	0.32
Liver, chick	10.12	2.90
Liver, guinea pig	6.15	1.95
Kidney, rat	1.26	0.65
Kidney, chick	0.65	0.23
Kidney, guinea pig	1.12	0.09
Spleen, rat	0.47	0.05
Spleen, chick	0.24	0.08
Spleen, guinea pig	0.18	0.01
Pancreas, chick	0.43	0.21
Muscle, rat	0.03	0.01
	μg./l.	μg./l.
Whole blood, rat, non-fasted	20	12
Whole blood, fasted 24 hr.	4.3	3.2
Whole blood, calf	9	2.6
Whole blood, chicken	51.5	18
Whole blood, sheep	6	1.4

"Crude x-methyl pteroylglutamic acid" was found to be a competitive antagonist for pteroylglutamic acid in a number of biological systems including *S. faecalis*, *L. casei*, rats, and chicks. The administration of the crude antagonist to rats produced a deficiency accompanied by leukopenia, agranulocytosis, and anemia with hyperplastic bone marrow changes. All the manifestations were prevented or reversed by increasing the folic acid content of the diet.[82] This is shown by the data in Table 3 from the studies

[80] G. J. Martin, L. Tolman, and J. Moss, *Arch. Biochem.* 12, 318 (1947).

[81] M. E. Hultquist and J. M. Smith, Jr., 1947, cited by Franklin *et al.*, ref. 80.

[82] A. L. Franklin, E. L. R. Stokstad, M. L. Belt, and T. H. Jukes, *J. Biol. Chem.* 169, 427 (1947).

acid, the "citrovorum factor," is concerned with many of the enzymatic functions of folic acid. Both folic acid and citrovorum factor appear to exist in natural materials, and it seems probable that an equilibrium between the two forms takes place in biological systems It is customary to measure folic acid activity by means of *S. faecalis* R, which responds to both folic acid and citrovorum factor. Assay with *L. citrovorum* may then be used to measure citrovorum factor activity, and this value may then be subtracted from the value obtained with *S. faecalis* to express the pteroylglutamic acid content of the material which is being investigated (Table 2).

TABLE 1

RESPONSE TO HOMOCYSTINE IN CHICKS DEFICIENT IN METHIONINE AND FOLIC ACID AS MODIFIED BY VARIOUS SUPPLEMENTS

(Twelve Chicks Were Used in Each Group The Depletion Period in the Basal Diet was 11 Days, and the Assay Period was 20 Days.)

Supplement per kilo of folic-acid-deficient diet	Gain, g	Response to			
		Homocystine	Homocystine + choline	Methionine	PGA
None	40				
DL-Homocystine, 6 g	54	14			
DL-Homocystine + choline, 2 g	126		86		
DL-Methionine, 4 g	101			61	
Pteroylglutamic acid (PGA), 5 mg	42				2
DL-Homocystine + PGA	82	40			28
DL-Homocystine + choline + PGA	181		139		55
DL-Methionine + PGA	116			74	15

IV. Folic Acid Antagonists and Their Effects on Blood Formation

The relation of a deficiency of folic acid to anemia and leukopenia in various species of animals made it evident even before the chemical structure of folic acid was known that, if it were possible to synthesize an "antagonist" of folic acid, such a substance might be expected to produce or accentuate anemia or leukopenia The discovery of the structure of the molecule of pteroylglutamic acid by Angier and co-workers[79] made it pos-

[77] L. S Dietrich, W J Monson, H Gwoh, and C A Elvehjem, *J. Biol Chem*

deficiency in pigs by Heinle, Welch, and co-workers[84-87] and by Cartwright, Wintrobe, and collaborators[88, 89] It was noted that the deficiency in the pigs was marked by an interference with growth and by the development of diarrhea and macrocytic anemia with megaloblastic hyperplasia of the bone marrow.

The field of investigation of folic acid antagonists was greatly enlarged by the synthesis of 4-aminopteroylglutamic acid, "Aminopterin"[90] Aminopterin was found by Franklin and co-workers[92] to be highly toxic to mice, and the toxic effects were not reversed by high levels of pteroylglutamic acid The average survival time of mice receiving one part of Aminopterin per million of purified diet was 6 days, with or without the addition of folic acid to the diet It was thus evident that the replacement of the hydroxyl group on the pteridine ring by NH_2 changed pteroylglutamic acid to a highly toxic compound, and a series of analogues of pteroylglutamic acid was synthesized which contained this modification of the molecule. The compounds included 4-amino-10-methyl pteroylglutamic acid (A-Methopterin), 4-aminopteroylaspartic acid (Amino-An-Fol), and others The use of these compounds was discussed by various investigators at a conference "Folic Acid Antagonists in the Treatment of Leukemia"[91]

The use of Aminopterin in the treatment of acute leukemia in children was first described by Farber and co-workers,[92] and temporary remissions were noted in ten of sixteen cases receiving the drug The evaluation of investigations of this type is difficult because of the tendency for spontaneous remissions to occur in the disease However, a considerable literature has accumulated describing the effects of Aminopterin and its chemical relatives in acute leukemia in children Clinical remissions are marked by a reduction in the number of blast forms in the bone marrow, a reduction in

[84] A D Welch, R W Heinle, R W Sharpe, W L George, and M Epstein, *Proc Soc Exptl Biol Med* 65, 364 (1947)

[85] R W Heinle, A D Welch, W L George, M Epstein, and J Pritchard, *J Lab Clin Med* 32, 1398 (1947)

[86] R W Heinle, A D Welch, and J A Pritchard, *J Lab Clin Med* 33, 1647 (1947)

[87] R W Heinle, A D Welch and H L Shorr, *J Lab Clin Med* 34, 1763 (1949)

[88] G L Cartwright, J Fay, B Tatting and M M Wintrobe, *J Lab Clin Med.* 33, 397 (1948)

[89] G E Cartwright, B Tatting, H Ashenbrucker, and M M Wintrobe, *Blood* 4, 301 (1949)

[90] D R Seeger, J M Smith, Jr, and M E Hultquist, *J Am Chem. Soc*, 69, 2567 (1947)

[91] Data from Conference on Folic Acid Antagonists in the Treatment of Leukemia, *Blood* 7, 97 (1952).

[92] S Farber, L K Diamond, R D Mercer, R E Sylvester, and J. A Wolff, *New Engl J Med* 238, 787 (1948)

by Franklin and co-workers. In this experiment a purified diet was used containing 1% succinylsulfathiazole and with pteroylglutamic acid omitted Weanling female rats received this diet, and by supplementing it with appropriate levels of pteroylglutamic acid and the crude antagonist it was readily possible to show a competitive reversal by pteroylglutamic acid of the effects of the antagonist in producing a specific syndrome. When pteroylglutamic acid was added to the diet at a level of 0.3 mg. or more per gram of antagonist, the rats were protected against pteroylglutamic acid deficiency However, if this ratio was widened the animals developed signs of acute folic acid deficiency which became progressively more intense as the pro-

TABLE 3

EFFECTS OF PTEROYLGLUTAMIC ACID (PGA) AND CRUDE ANTAGONIST ON HEMATOLOGY OF RATS FED PURIFIED DIET PLUS SUCCINYLSULFATHIAZOLE (FRANKLIN *et al.*)[32]

Group	Supplement per kilo of diet		Hemoglobin, g./100 ml			White blood cells per cu mm × 10⁻³		Granulocytes per cu mm		Gain in wt., 4 wk
	PGA, mg	Antagonist, g	1 wk	2 wk	3 wk	3 wk	5 wk	3 wk	5 wk	
1	1	0	14.5	16.6	19.3	10.5	15.2	2400	2600	71
2	*0*	*0*	*14.0*	*15.1*	*18.3*	*8.2*	*12.6*	*1500*	*1500*	66
3	0	1	14.5	13.6	10.5	2.8	0.8	84	16	29
4	0	10	15.4	10.5	*	0.8	*	8	*	17
5	1	10	15.4	12.2	*	2.5	*	100	*	22
6	10	10	13.8	15.1	20.2	9.5	14.2	1200	1100	79
7	3	1	15.8	15.1	20.9	10.9	13.7	1500	1100	86
8	100	10	16.0	15.7	20.6	15.5	18.2	2900	1600	74

* All animals dead

portion of antagonist was increased and which were marked by slowing of growth, anemia, leukopenia, and granulocytopenia. The investigation made it evident that the rat is extraordinarily sensitive to the development of granulocytopenia upon administration of the crude antagonist Severe diarrhea is observed in the experimental animals together with necrotic and ulcerative changes in the oral cavity and chromodacryorrhea with encrusted vibrissae. It was suggested that the antagonist "might be used experimentally in an attempt to modify blood dyscrasias marked by erythrocytosis or leukocytosis " Clinical experiments[33] indicated, however, that the crude antagonist had very little biological potency for human subjects.

The antagonist was used extensively for the production of folic acid

[33] R. W Heinle and A D Welch, *J Clin Invest* 27, 539 (1948)

marked by ulcerative stomatitis, gastric distress, nausea, vomiting, diarrhea, alopecia, and deafness These changes have shown some tendency to be reversed by leukovorin if this substance is administered promptly

The effects of injected Aminopterin on rats and mice were studied by Philips and Thiersch,[106] who found that the LD_{50} was 1 9 ± 0 3 mg per kilo of body weight for mice and 4 5 ± 1 4 mg. for rats Severe watery diarrhea appeared within 2 days after fatal doses of the drug were administered, and blood was passed during the terminal stages Progressive weight losses were noted following the first day in rats receiving 40 mg. per kilo with changes in the femoral marrow starting at 12 hr and liquefaction with disappearance of the hemopoietic tissues becoming complete by 72 hr Destructive changes occurred in the intestinal tract, starting with venous hyperemia and followed by extensive desquamation of surface and crypt epithelium Only moderate effects were produced on lymphoid tissues which is in contrast to the toxic action of the nitrogen mustards Higgins[107] has also reported pathological effects including adrenal hyperplasia and atrophy of the thymus in rats The intestinal changes in rats and mice following the administration of Aminopterin are accompanied by a decrease in the rate of incorporation of formate in the nucleic acids in the tissue of the wall of the small intestine [108, 109] When both C^{14}-formate and N^{15}-adenine were administered, Aminopterin decreased the incorporation of formate into the intestinal desoxyribonucleic acid, adenine, guanine, and thymine to 6 to 8% of the control levels while decreasing that of the ribonucleic acid, adenine, and guanine to 32% of the control values These findings may indicate that Aminopterin inhibits cellular proliferation by blocking the synthesis of desoxyribonucleic acid, especially by inhibiting the incorporation of the single carbon fragment into the methyl group of thymine and the 2 and 8 positions of the purine ring

V. Vitamin B_{12} in Nutrition and Hematopoiesis

1 Vitamin B_{12} Deficiency in Experimental Animals

Experiments in the early 1930's[110-114] with rats on certain simplified diets containing only vegetable sources of the vitamin B complex showed

[106] F S Philips and J B Thiersch, *J Pharmac*[illegible] 95, 303 (1949)
[107] [illegible]
[108] [illegible] *ancer Research* 10, [illegible]
[109] D A Goldthwait and A Bendich, *Federation Proc* 10, 190 (1951)
[110] K H Coward, K M Key, and B G E Morgan, *Biochem J.* 23, 695 (1929)
[111] K H Coward, K M Key, F. J Dyer, and B G E Morgan, *Biochem J.* 24, 1952 (1930), 25, 551 (1931)
[112] K H Coward, F. J Dyer, R. A. Morton, and J H Gaddum, *Biochem J.* 25, 1102 (1931)

the total cellularity in the marrow and a period of regeneration with a gradual return to the normal percentage of granulocytic and erythrocytic elements. Changes in the blood picture include a return to normal level both in patients with initially high white counts and also in those who were leukopenic The percentage of immature cells and blast forms in the blood

TABLE 4

REMISSIONS WITH 4-AMINOPTEROYLGLUTAMIC ACID (AMINOPTERIN), 4-AMINO-10 METHYLPTEROYLGLUTAMIC ACID (A-METHOPTERIN), AND 4-AMINOPTEROYLASPARTIC ACID (AMINO-AN-FOL), IN ACUTE LEUKEMIA, PRINCIPALLY IN CHILDREN

No of cases	No of remissions	Reference
10	2	W. Jacobson, *et al.*[93]
8	5	M. Pierce and H. Alt[94]
54	8	J M. Stickney, *et al.*[95, 96]
	10%	S. Farber[97]
35	9	W. Dameshek[98]
8	3	*Jimenez de Asaa*[99]
43	4	L. M. Meyer, *et al.*[100]
250	30%	J B. Thiersch and F. S. Philips[101]
14	2	M S. Sacks, *et al.*[102]
9	5	*C. H Smith and W. R Bell*[103]
13	9	J. V. Dacie, *et al.*[104]
27	6	J F. Wilkinson and C Gardikas[105]

stream decreases markedly. However, these changes in the direction of normality soon become reversed and are followed by fatal relapses The results obtained by various investigators are summarized in Table 4.

Toxic symptoms following the use of Aminopterin in human subjects are

[93] W Jacobson, W C. Levin, and G Holt, *J Lab Clin Med* **33**, 1641 (1948)

[94] M Pierce and A Alt, *J Lab. Clin Med.* **33**, 1642 (1948).

[95] J M Stickney, A B Hagedorn, S D Mills, and T. Cooper, *J. Clin Invest* **33**, 1481 (1948)

[96] J M Stickney, S D Mills, A B Hagedorn, and T. Cooper, *Proc Staff Meetings Mayo Clinic* **24**, 525 (1949)

[97] S Farber, *Blood* **4**, 160 (1949)

[98] W Dameshek, *Blood* **4**, 168 (1949)

[99] F. Jimenez de Asaa, *Rev soc argentina Hemat* **1**, 353 (1949)

[100] L. M. Meyer, H Fink, A Savitsky, M Rowen, and N. D Ritz, *Am J. Clin Path* **19**, 119 (1949)

[101] J B Thiersch and F S Philips, *Am J Med Sci* **217**, 575 (1949).

[102] M. S. Sacks, G T Bradford, and E B Schoenbach, *Ann. Internal Med* **32**, 80 (1950).

[103] C H. Smith and W R. Bell, *Am J. Diseases Children* **79**, 1031 (1950)

[104] J V. Dacie, E Dresner, D. L Molbin, and S C White, *Brit. Med. J.* **1**, 1447 (1950)

[105] J F Wilkinson and C Gradikas, *Lancet* **1**, 325 (1951)

Concentrated anti-pernicious-anemia liver extract was shown to be an excellent source of the animal protein factor.[124] Finally, in 1948, it was shown that the anti-pernicious-anemia factor was produced by fermentation when a microorganism isolated from hen feces was grown in pure aerobic culture.[125] Simultaneously, crystalline vitamin B_{12} was found to have animal protein factor activity in chicks on all-vegetable diets.[126]

In contrast to the early researches with folic acid, which was shown to be needed for the formation of red and white blood cells as well as for growth in various experimental animals, vitamin B_{12} was related far more prominently to early growth of the young animal than to hematopoiesis. The importance of the vitamin to the offspring in the very early stages of life was shown by experiments on vitamin B_{12} deficiency in pregnant and lactating rats; it was found that a high mortality, associated with uremic poisoning, was found in suckling rats when their mothers were deficient in vitamin B_{12}.[127, 128] Similarly it was observed that the hatchability of eggs laid by hens on diets deficient in the animal protein factor was low and that the embryos showed specific deformities during development.[129] Even when chicks were successfully hatched from such eggs, the mortality of the animals was high during the first few days of life.

2. Vitamin B_{12} and Cobalt Deficiency in Ruminants

A deficiency of inorganic cobalt in the soil produces a wasting disease in ruminants which has been described as occurring in various areas in various parts of the world. Non-ruminants, such as horses and rabbits, can subsist on forage crops grown in the deficient areas and can reproduce without signs of dietary disturbance. Cobalt deficiency in ruminants is marked by listlessness, a failure in appetite, lethargy, weakness, and progresses to a fatal termination. Marston[130] has commented that the "hemoglobin content of the blood has been observed to fall to less than half the normal of about 14 volumes percent of oxygen in sheep, and in extreme cases, an oxygen carrying capacity of less than 3 volumes percent of oxygen is often encountered."

[124] C. A. Nichol, A. R. Robblee, W. W. Cravens, and C. A. Elvehjem, *J. Biol. Chem.* 170, 419 (1947).
[125] E. L. R. Stokstad, A. C. Page, Jr., J. Pierce, A. L. Franklin, T. H. Jukes, R. W. Heinle, M. Epstein, and A. D. Welch, *J. Lab. Clin. Med.* 33, 860 (1948).
[126] W. H. Ott, E. L. Rickes, and T. R. Wood, *J. Biol. Chem.* 174, 1047 (1948).
[127] T. F. Zucker and L. M. Zucker, *Abstracts* 110th Meeting, American Chemical Society, p. 7B (1946).
[128] M. O. Schultze, *Proc. Soc. Exptl. Biol. Med.* 72, 613 (1949).
[129] O. Olcese, J. R. Couch, J. H. Quisenberry, and P. B. Pearson, *J. Nutrition* 41, 423 (1950).
[130] H. R. Marston, *Ann. Rev. Biochem.* 8, 570 (1939).

that a deficiency was produced during pregnancy and lactation and that the deficiency could be prevented or remedied by feeding fresh liver or liver extract. These investigations were continued for many years by Cary, Hartman, and their collaborators[115, 116, 117] in the United States Department of Agriculture laboratories at Beltsville, and these workers showed that the protective factor "factor X," was present in such natural materials as whole milk, cheese, and liver extract but was absent from yeast. Indeed, *the addition of yeast to the diet served to accentuate the deficiency in rats.* Cary and Hartman[116] also noted that extraction with hot alcohol removed factor X from commercial casein and that the deficiency could be made more acute in rats by adding hot-alcohol-extracted casein, soybean meal, or other sources of vegetable protein to the basal diet. This observation indicated that vitamin B_{12} was needed for the metabolism of proteins at some stage, for a deficiency of factor X was accentuated by raising the protein level in the diet. Cary and Hartman[117] found it possible to concentrate factor X from anti-pernicious-anemia liver extract by precipitation with ammonium sulfate so that the factor was present in amounts detectable with the rat assay when only a few micrograms of the ammonium *sulfate precipitate of concentrated liver extract was added to the diet.*

In the meantime, a parallel series of investigations with chicks had shown that low hatchability and slow growth were obtained when diets were fed containing only vegetable sources of protein. The protective factor, "animal protein factor," was present in sardine fishmeal, fish solubles, liver meal, cow manure, and rumen contents.[118-122] Moreover, the animal protein factor was found to be present in the litter of chicken houses, in which source it had been formed by bacterial fermentation in the chicken droppings after they had been voided. The temperature of the hen house was found to play a part in this fermentation, and the low hatchability associated with the winter months was found to be caused by a diminution in the production of the animal protein factor in the fermentation taking place in the litter.[123]

[113] L W Mapson, *Biochem J* **26**, 970 (1932)
[114] L W Mapson, *Biochem J* **27**, 1061 (1933)
[115] A M Hartman, L P Dryden, and C A Cary, *J Biol Chem* **140**, liv (1941)
[116] C A Cary and A M Hartman, Yearbook of Agriculture, 1943–1947, U S Department of Agriculture, 1947, p 779
[117] C A Cary, A M Hartman, L P Dryden, and G D Likely, *Federation Proc* **5**, 128 (1946)
[118] T C Byerly, H W Titus, N R Ellis, and R B Nestler, *Poultry Sci* **16**, 322 (1937)
[119] R B Nestler, T C Byerly, N R Ellis, and H W Titus, *Poultry Sci* **15**, 67 (1936)
[120] W W Cravens, W H McGinnon, and J G Halpin, *Poultry Sci* **24**, 305 (1945)
[121]
[122]
[123]

necessary to produce a satisfactory clinical response and maintenance appeared to be in the neighborhood of 1 to 2 μg injected daily. Furthermore, just as in the case of concentrated liver extracts, vitamin B_{12} was found to be of very low effectiveness when administered orally unless normal human gastric juice or some other source of the intrinsic factor was simultaneously given by mouth[22] The oral effectiveness of vitamin B_{12} in pernicious anemia, like that of concentrated liver extract, varies greatly in different patients This is due to the fact that some patients with pernicious anemia still secrete small amounts of the intrinsic factor and hence are able to utilize vitamin B_{12} to a limited extent. However, other patients are almost completely without the intrinsic factor, and more massive doses of the vitamin administered by mouth are needed to produce a clinical response. It appears that a single oral dose of 3 mg of vitamin B_{12} will, in most cases, serve to produce a good remission in a case of pernicious anemia in relapse[43] If such effects can be consistently produced, it may well be that oral treatment with large doses of vitamin B_{12} will eventually prove to be the routine treatment for pernicious anemia

Sensory and motor disturbances which are associated with changes in the posterior columns and pyramidal tracts of the spinal cord may occur in untreated or relapsed cases of pernicious anemia These changes may progress to an irreversible state if untreated, but improvements may be produced in the patient by injecting vitamin B_{12} It was reported by Mueller and co-workers[118] that 5 μg on alternate days produced a rapid response in cases in which the nervous symptoms were of recent origin but that the same dosage had little or no effect in a patient who had suffered from cord lesions for more than 2 years and a daily injection of 10 μg for combined system disease was suggested In another report by Kinnear and Hunter[119] it was found that, when the nerve damage had a duration of more than 18 months, no response was obtained to an average weekly dose of 40 to 60 μg of vitamin B_{12} Evidently, the use of generous dosage of the vitamin in treating combined system disease is to be recommended

Investigations with liver extract have shown that a great deal of variability exists in the time between cessation of therapy and the onset of relapse in pernicious anemia It is evident that vitamin B_{12}, in contrast to [illegible]

[118] J F Mueller, T Jarrold, V. R. Hawkins, and R. W. Vilter, *Ohio State Med J* 46, 225 (1950)

[119] T Kinnear and R. B Hunter, *Edinburgh Med J*. 57, 65 (1950)

[120] L. A. Erf and B Weiner, *Blood* 4, 845 (1949)

As long ago as 1933 it was noted by Filmer[131] that whole liver would cure the disease of cattle and sheep caused by cobalt deficiency, although Filmer and Underwood[132] found in 1937 that liver contained insufficient cobalt to account for its therapeutic effect. Although effective when given by mouth, cobalt was not active when injected in treating the deficiency in sheep, thus leading to the suggestion that cobalt exerted its beneficial effect by acting on some of the microorganisms in the rumen.[133, 134] Accordingly, when vitamin B_{12} was found to contain cobalt, it appeared probable that the function of cobalt in ruminant nutrition was to enable the synthesis of the vitamin to take place in the rumen, and various investigations have now indicated that this indeed is probably the case, although early experiments[135, 136] appeared to show that vitamin B_{12} when injected was ineffective in alleviating the deficiency. It appeared subsequently that the failures to obtain a response were due to underdosage, and that the vitamin B_{12} requirement of ruminants was comparatively high when measured against the requirement of human pernicious anemia patients. Improvements in appetite and increases in hemoglobin level and in growth rate have been now reported to be produced by injecting suitable quantities or feeding similar quantities of vitamin B_{12} to cobalt-deficient lambs.[137] With the recognition of the importance of cobalt in ruminant nutrition and with the knowledge of the extent and locality of cobalt-deficient soil areas, the practice of adding cobalt when necessary to mineral mixtures for ruminants has become widespread, so that cobalt deficiency has greatly decreased both in Australia and the United States by the use of suitable salt mixtures in feeding ruminants Simultaneously with the recognition that vitamin B_{12} can be readily produced by microbial growth, the use of suitable fermentation materials in animal feeds has become widespread and vitamin B_{12} deficiencies in "all-vegetable" diets for pigs and poultry are now easily remedied without recourse to products of animal origin.

3 Vitamin B_{12} in the Treatment of Pernicious Anemia

The use of vitamin B_{12} in pernicious anemia, as was to be anticipated, led to a repetition of the observations which had been made over a period of twenty years in clinical experiments with concentrated injectable liver extracts The crystalline vitamin upon injection was found to produce hemopoietic remissions in pernicious anemia and an amelioration of the neurological symptoms and glossitis associated with this disease The amount

131 [illegible]
132 [illegible] (1937).
133 [illegible]
134 [illegible] 315 (1944)
135 [illegible]
136
137

choline, it was found that either vitamin B_{12}, choline, or methionine would produce a growth effect in chicks on the diet and that these growth effects were not supplementary so that combinations of vitamin B_{12} with choline or betaine produced no greater growth than the substances when fed separately. Similar findings were reported at about the same time by Schaefer and co-workers.[145]

A relationship between vitamin B_{12} deficiency and the utilization of homocystine in chicks on methionine-deficient diets was described by Jukes and co-workers.[146] Homocystine did not promote the growth of vitamin-B_{12}-deficient chicks on a purified diet consisting of glucose, alcohol-extracted soybean protein, cystine, dimethylaminoethanol, minerals, and vitamins, but the chicks responded to a supplement of methionine. However, if the chicks received vitamin B_{12} they responded to either methionine or homocystine. Further investigations, some of the results of which are shown in Table 5, show that the response to methionine was affected very little by vitamin B_{12} or folic acid. There was no growth response to homocystine unless vitamin B_{12} was also added, and the responses to mixtures of homocystine and betaine or homocystine and choline in the absence of vitamin B_{12} were no greater than the responses to betaine or choline when added singly. Evidently chicks, like rats, can utilize certain amino acids as sources of the methyl group of methionine synthesis when vitamin B_{12} is supplied, but in the absence of vitamin B_{12} a source of "labile methyl," such as choline or betaine, is required for the methylation of homocystine.

The requirement for vitamin B_{12} by rats, mice, and chicks is increased by adding thyroid hormone to the diet. The mechanism of this effect is unknown, although it has been suggested by Rupp and co-workers[147] that vitamin B_{12} can decrease the loss of nitrogen resulting from the catabolic action of thyroxine in force-fed rats on constant food intake. The use of thyroid-supplemented diets in the assay of the vitamin B_{12} content of natural materials with rats and mice has been adopted by various investigators.[148, 149, 150] However, Ershoff[151] reported that liver residue contained a factor other than vitamin B_{12} which counteracted the growth-retarding effect of thyroid powder when added to purified diets fed to rats

[145] A E Schaefer, W D Salmon, and D R Strength, *Federation Proc* 8, 395 (1949)

[146] T H Jukes, E L R Stokstad, and H P Broquist, *Arch Biochem.* 25, 453 (1950)

[147] J Rupp, K. E Pashkis, and A Cantarow, *Proc Soc Exptl Biol Med* 76, 432 (1951)

[148] J J Bethell and H A Lardy, *J Nutrition* 37, 495 (1949)

[149] U D Register, V J Lewis, and C A Elvehjem, *Proc Soc Exptl. Biol Med.* 71, 167 (1949)

[150] G A Emerson, *Proc Soc Exptl Biol Med* 70, 392 (1949)

[151] B H Ershoff, *Arch Biochem* 28, 359 (1951)

days Great variations, of course, may be expected among individual patients as to the length of time a remission will last upon withdrawal of therapy

Vitamin B_{12} appears to be comparatively well utilized when given by mouth to normal animals, and it may be presumed that the vitamin is efficiently utilized when given orally to normal human subjects who are not suffering from a deficiency of the intrinsic factor in their gastric juices Bethell and co-workers[141] have recently described a patient who was suffering from a dietary deficiency of vitamin B_{12} due to an improper food intake The patient had free hydrochloric acid in his gastric secretions, and his deficiency was cured by giving 20 μg of vitamin B_{12} daily by mouth.

4 Metabolic Changes Involving Vitamin B_{12}

One of the first observations relating vitamin B_{12} to a specific role in metabolism was made by Patton and co-workers,[142] who found that methionine produced a marked growth response in chicks when added at a level of 0 15% to a basal all-vegetable diet consisting mainly of corn and soybean meal Lower levels of methionine had very little effect upon growth, but, when the basal diet was modified to contain 2% of sardine fishmeal which supplied only 0 04% of additional methionine to the diet, growth was rapid and further growth responses could not be obtained when free methionine was added at levels of 0 15% and 0 30% This showed that the fishmeal contained a factor which had a sparing action on the methionine requirement of chicks Similar effects may be produced by vitamin B_{12} on such diets A relation of vitamin B_{12} to methionine was shown by experiments with *E coli* reported by Shive,[143] who found that the inhibitory effect of sulfanilamide on this organism was overcome by the addition to the culture medium of either vitamin B_{12} at a level of 0 3 μg per liter or methionine at a level 300,000 times as high This appeared to show that the formation or utilization of vitamin B_{12} by *E coli* was suppressed by the addition of sulfanilamide to the medium and that this suppression could be overcome either by supplying the preformed vitamin or by adding methionine, the latter being the product of the reaction which was catalyzed by vitamin B_{12}

An interchangeability between vitamin B_{12}, choline, and betaine was reported by Gillis and Norris[144] in experiments with chicks on a diet of cereals and soybean meal Although this diet was by no means deficient in

[141] F. H Bethell M E Swendseid, S Miller and A A Cintron Rivera, *Ann Int Med* **35**, 518 (1951)

[142] A R Patton, J P Marvel, H G Petering, and J Waddell, *J Nutrition* **31**, 485 (1946)

[143] W Shive, *Ann N Y Acad Sci* **52**, 1212 (1950).

[144] M B Gillis and L C Norris, *J Biol Chem* **179**, 487 (1949)

and further studies on the relationship of vitamin B_{12} and folic acid in tyrosine metabolism need to be made

VI. The Citrovorum Factor

During an investigation of amino acids by microbiological assay procedures it was noted by Sauberlich and Baumann[159] that the lactic acid organism *Leuconostoc citrovorum* 8081 needed an unidentified growth factor which was present in liver extract and yeast The organism could be made to grow by additions of thymine or of high levels of pteroylglutamic acid to the culture medium, but it was evident that much more rapid growth was obtained in response to an unidentified "citrovorum factor" (CF) which was present in liver extract Comparative assay of natural source materials with chicks and *Le citrovorum* showed that CF could be differentiated from vitamin B_{12} The two factors were also shown to be different in that they migrated in opposite directions in an electric field[160] and that CF was stable to alkali under conditions in which vitamin B_{12} was destroyed[161] Broquist and co-workers[162] suggested a functional relationship between *thymidine, folic acid, and CF based upon a synergistic effect of thymidine* on pteroylglutamic acid in promoting growth of the organism

Further investigations by Sauberlich[163] showed that concentrates containing CF would reverse the inhibitory effect of Aminopterin for *Le. citrovorum* and that the administration of large doses of folic acid to rats or to a human subject increased as much as 200-fold the urinary excretion of CF[164] These important findings suggested strongly that CF was a biologically functional derivative of pteroylglutamic acid

It was reported by Bardos and co-workers[165] and by Broquist and co-workers[166] that CF, which was renamed "folinic acid" by the Texas investigators, was quite labile to dilute hydrochloric acid, the action of which was to transform it to a new compound which was no longer active for *Le citrovorum* but retained its activity for *S faecalis* "Folinic acid" was more active than pteroylglutamic acid in reversing the inhibition of the folic

[159] H E Säuberlich and C A Baumann, *J Biol Chem* 176, 165 (1948).

[160] C N Lyman and J M Prescott, *J Biol Chem* 178, 523 (1949)

[161] K A Lees and W B Emery, *Biochem J* 45, ii (1949)

[162] H P Broquist, E. L R Stokstad, C E Hoffmann, M L Belt, and T H Jukes, *Proc Soc Exptl Biol Med* 71, 549 (1949)

[163] H L Säuberlich, *Federation Proc* 8, 247 (1949)

[164] A L Franklin, E L R Stokstad, C E Hoffmann, M L Belt, and T. H Jukes, *J Biol Chem* 181, 467 (1949)

[165] T. J Bardos, T J Bond, J Humphreys, M Sibley, and W Shive, *J. Am. Chem Soc* 71, 3852 (1949)

[166] H P Broquist, E L R Stokstad, and T. H. Jukes, *J Biol. Chem.* 185, 399 (1950)

The relation of vitamin B_{12} and folic acid to the metabolism of choline and methionine may indicate that these vitamins are concerned in the prevention of fatty livers on diets which supply borderline quantities of choline Popper and co-workers[152] found that vitamin B_{12} inhibited the development of histologic changes following the administration of a toxic dose of carbon tetrachloride to rats, while György and Rose[153] noted that vitamin B_{12} had a lipotropic effect in rats. In studies with dogs on a purified diet low in methionine, it was found that severe edema and ascites were developed which could be prevented by adding a concentrate of vitamin B_{12} or by increasing the choline content of the diet.[154] A relation of vitamin B_{12} to fatty livers in dogs was suggested by the experiments of Burns and McKibbin[155] Dogs on a purified diet deficient in choline but containing 19% casein grew slowly and in many cases developed fatty livers. The injection of vitamin B_{12} was found to produce reductions in liver fat based on biopsy samples

TABLE 5

Weights of Chicks at 25 Days on Purified Diet (Glucose + 25% Soybean Protein) with Various Supplements

Vitamin addition per kilo of diet	Additions per kilo of diet						
	None	6 g DL-homocystine	2 g betaine	2 g choline	Homocystine + betaine	Homocystine + choline	2 g DL-methionine
None	75	74	108	141	119	140	176
5 mg PGA	92	88		155	144	151	202
50 μg B_{12}	182	242		233	296	315	312
B_{12} + PGA	255	321		255	338	324	349

Disturbances in tyrosine metabolism have been observed in pernicious anemia[156] leading to an increased urinary excretion of total phenolic compounds A decrease in the ratio of hydroxyphenyl acids to ether-soluble phenols insoluble in $NaHCO_3$ was noted in three of four pernicious anemia patients during the progress of remission under treatment with vitamin B_{12} Folic acid has also been related to the oxidation of tyrosine,[157, 158]

152 H Popper, D. Koch-Wester, and P B Szanto, *Proc. Soc Exptl Biol Med* **71**, 688 (1949)

153 [illegible] and C. S. Rose, *Proc. Soc. Exptl. Biol. Med.* **73**, 372 (1950).

154 [illegible] 3, 201 (1951)

155 [illegible]

156 [illegible] *Med* **32**, 1242 (1947).

157 G. Rodney, M E Swendseid, and A L Swanson, *J. Biol. Chem.* **168**, 395 (1947)

158 C. W. Woodruff and W. J Darby, *J Biol. Chem* **172**, 851 (1948)

Welch and Nichol[171] found that Aminopterin inhibited the production of CF activity by rat liver slices in the presence of folic acid and that Aminopterin also stopped the urinary excretion of CF by rats even when pteroylglutamic acid was also given These findings indicate that Aminopterin blocked the enzymatic conversion of pteroylglutamic acid to CF However, it also appears that Aminopterin can block the action of CF in the cell and that this action is concerned with the formation of thymidine, for it has been shown that CF competitively, and thymidine non-competitively, reverse the inhibitory effects of Aminopterin for *S faecalis*, *Le citrovorum*, and *E coli* [166, 172, 173, 174]

Interest was soon shown in the relation of CF to the antileukemic effects of the Aminopterin group of folic acid antagonists It was found by Burchenal and co-workers[175] that the effect of 4-amino-10-methyl pteroylglutamic acid in postponing the death of mice with transplanted leukemia AK4 was blocked by prior administration of leucovorin, 0 75 mg of this substance reversed the antileukemic effect of 2 mg of the antagonist However, if the antagonist was injected 1 hr prior to the administration of leucovorin, the effectiveness of the latter was diminished Conversely, although pteroylglutamic acid is ineffective when given simultaneously in overcoming the toxic effects of Aminopterin in mice, it became slightly active if given several hours prior to giving the antagonist [176] This observation would appear to indicate that some CF was formed from pteroylglutamic acid in the tissues during the period prior to the administration of the antagonist

The use of leucovorin to reverse the toxic effects of Aminopterin and its derivatives in patients under treatment with these antagonists has been described by several clinical investigators Schoenbach and co-workers[177] used leucovorin to combat the toxic effects of Aminopterin and A-Methopterin when these substances were being used for the attempted treatment of metastatic neoplasms One patient was given A-Methopterin, 10 mg daily, and developed ulcerated lesions in the mouth together with leukopenia The administration of leucovorin, 6 mg daily, was followed by healing of the ulcers in 5 days and by an increase in white blood cell count The ulcers reappeared 10 days after cessation of the treatment with leu-

[171] C A Nichol and A D Welch, *Proc Soc Exptl Biol Med* **74**, 403 (1950)
[172] H E Sauberlich *J Biol Chem* **195**, 337 (1952)
[173] W Shive *Abstracts*, 117th meeting, American Chemical Society, p 1C (1950)
[174] A L Franklin, E L R Stokstad, C E Hoffmann, M L Belt, and T H Jukes, *J Am Chem Soc* **71**, 3549 (1949)
[175] J H Burchenal, G M Babcock, H P Broquist, and T H Jukes, *Proc Soc Exptl Biol Med* **74**, 735 (1950)
[176] L N Greenspan, A Goldin, and E B Schoenbach, *Cancer* **4**, 619 (1951)
[177] E B Schoenbach E N Greenspan, and J Colsky, *J Am Med Assoc* **144**, 1558 (1950)
[178] J H Burchenal and L M Kingsley-Pillers, *J. Clin Invest* **30**, 631 (1951)

acid antagonist "x-methyl pteroylglutamic acid" for *S. faecalis* R.[167] Broquist and co-workers[168] showed that concentrates of CF prepared from beef liver were able to protect mice against the toxic effects of Aminopterin (Table 6) and were able to prevent folic acid deficiency in chicks on purified diets

A relationship between ascorbic acid, pteroylglutamic acid, and CF was shown by Nichol and Welch,[169] who found that ascorbic acid accelerated the formation of CF from pteroylglutamic acid *in vitro* by rat liver slices The amount of CF activity which was produced after incubation of the slices for 2 hr was doubled when 100 μg. of pteroylglutamic acid or 10 mg of ascorbic acid was added per gram of tissue, but a mixture of pteroylglutamic and ascorbic acids increased the level of CF three- to fivefold It

TABLE 6

EFFECTS OF PTEROYLGLUTAMIC ACID (PGA), CITROVORUM FACTOR (CF) AND 4-AMINOPTEROYLGLUTAMIC ACID (AMINOPTERIN) ON GROWTH AND SURVIVAL OF MICE ON PURIFIED DIET CONTAINING NO ADDED PGA

Group no	Supplements injected per mouse three times weekly	Weight and no surviving (in parentheses)		
		1 day	5 days	9 days
1	None	19 3 (12)	20 2 (12)	23 0 (12)
3	10 μg Aminopterin	19 5 (12)	17 3 (3)	(0)
4	20 μg Aminopterin	20 0 (12)	16 3 (3)	(0)
6	10 μg Aminopterin + 20 μg PGA	18 0 (12)	16 2 (4)	(0)
7	20 μg Aminopterin + 20 μg. PGA	19 2 (12)	15 0 (5)	(0)
9	10 μg Aminopterin + 100,000 U CF*	19 3 (12)	20 5 (12)	19 5 (12)
10	20 μg Aminopterin + 100,000 U CF*	19 3 (12)	18 0 (8)	15 7 (3)

* Equivalent to 20 μg of folic acid activity

was also found that the urinary excretion of CF following the administration of a dose of pteroylglutamic acid was found increased by giving simultaneously an oral dose of ascorbic acid

Broquist[170] has employed resting cells of a strain of *S faecalis* which is resistant to the inhibitory effects of 4-amino-10-methyl pteroylglutamic acid These cell preparations are able to produce CF activity when incubated with *pteroylglutamic acid, and the production of CF is increased* by adding ascorbic acid, formate, or serine Some typical results are summarized in Table 7

[167] T J Bond, T J Bardos, M Sibley, and W Shive, *J Am Chem Soc* 71, 3852 (1949)

[168] H P Broquist, E L R Stokstad, and T H Jukes, *Federation Proc* 9, 156 (1950)

[169] C A Nichol and A D Welch, *Proc Soc Exptl. Biol Med* 74, 52 (1950)

[170] H. P. Broquist, unpublished data

to yield several acid transformation products. At pH 1 3 or below, isoleucovorin chloride was obtained, $C_{20}H_{22}N_7O_6Cl$ This could be recrystallized by solution in 12 *N* hydrochloric acid and dilution to 2 *N*. Crystallization of this compound at pH 2 from 0 01 *N* hydrochloric acid yielded anhydroleucovorin-A, $C_{20}H_{21}N_7O_6 \cdot 4H_2O$, and this compound at pH 4 gave anhydroleucovorin-B, $C_{20}H_{21}N_7O_6 \cdot \frac{1}{2}H_2O$. Biological assay of these compounds indicated that their activity for *Le citrovorum* was extremely low, but they retained full activity for *S faecalis* It seems probable that the CHO group may form a CHOH or CH bridge between the 5 and 10 positions when leucovorin is treated with acid, thus yielding a series of tetrahydropteroylglutamic acid derivatives The sensitivity of these compounds

TABLE 7

ENZYMATIC FORMATION OF CF BY RESTING CELLS OF A STRAIN OF *S faecalis*

Supplements			
PGA	Ascorbate	Other addition	Results, CF activity
			mμg
—	—	—	0 8
—	5 mg	—	0 8
—	—	10 mg formate	0 8
—	5 mg	10 mg formate	0 6
1 μg	—	—	21
1 μg	5 mg	—	160
1 μg	—	10 mg formate	400
1 μg	5 mg	10 mg formate	510
1 μg	5 mg	3 mg DL-serine	550
Boiled cells			
1 μg	5 mg	10 mg formate	0 6

to pH changes makes it difficult to examine them individually with respect to biological activity However, it was found by Cartwright and co-workers[154] that acid-treated leucovorin was about one-tenth as active as leucovorin in reversing Aminopterin toxicity in mice and that when administered to patients with pernicious anemia in relapse the acid-treated product was also active but less so than the starting material. Isoleucovorin chloride and anhydroleucovorins A and B are converted to leuco-

biological transfer of single-carbon fragments, suggest the possibility of an equilibrium between leucovorin and pteroylglutamic acid in biological

[154] G E Cartwright, M M Wintrobe, H F Broquist, and T. H Jukes, *Proc Soc Exptl Biol Med* 78, 563 (1951)

covorin Similar results were noted with a second patient receiving 2 mg. of Aminopterin daily. Burchenal and Kingsley-Pillers[178] found that the tolerance for A-Methopterin appeared to be greatly increased by the administration of leucovorin. A patient who received 2.5 mg. of the antagonist daily for 8 days developed toxic mouth ulcerations and leukopenia. However, the patient was able to tolerate doses of 45 to 60 mg. daily for 21 days when 3 mg of the leucovorin was given simultaneously. Other investigators have also discussed the effect of leucovorin in relieving the toxicity produced by the Aminopterin group of folic acid antagonists[11]

1. Chemistry

It is possible to prepare CF from pteroylglutamic acid by formylation and reduction. The preparation of a biologically active reaction mixture by this means was described by Shive and co-workers.[179] Brockman and co-workers[180] described the crystallization of a compound "leucovorin" which was prepared from pteroylglutamic acid by catalytic reduction with hydrogen and platinum in folic acid at 0° to 30°, followed by adsorption of impurities with Magnesol at pH 7, adsorption of activity on Darco G-60 at pH 4, elution, fractional crystallization of the barium salt, and finally chromatography on columns of Magnesol The barium salt had a composition corresponding to $C_{20}H_{21}N_7O_7Ba \cdot 5H_2O$. The ultraviolet absorption spectrum of leucovorin in 0.1 *N* sodium hydroxide solution showed a maximum at 282 mμ and minimum at 243 mμ. The substance had biological properties corresponding to those of CF; 0.15 mμg of the anhydrous free acid corresponded to 1 CF "unit" Leucovorin competitively reversed the lethal effects of Aminopterin in mice, and about 30 μg of leucovorin prevented the toxicity of 10 μg of Aminopterin when the two substances were injected simultaneously Leucovorin was decomposed on standing at pH 2 at room temperature, and the resultant product was almost inactive in the *Le citrovorum* test but retained folic acid activity for *S faecalis* and *L casei*.

Chemical studies indicated that Folinic Acid S F. and leucovorin have a structure corresponding to 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid [181, 182]

The treatment of leucovorin with dilute acid, which has been noted to modify its biological activity, was found by Cosulich and co-workers[182]

[179] W Shive, T J Bardos, T J Bond, and L L Rogers, *J Am. Chem Soc* 72, 2818 (1950)

[180] J. A Brockman, Jr , B Roth, H P Broquist, M E Hultquist, J. M Smith, Jr , M J Fahrenbach, D. B Cosulich, R P Parker, E L. R Stokstad, and T H Jukes, *J Am Chem Soc* 72, 4325 (1950)

[181] E H Flynn, R G Jones, and W Shive, *Abstracts* 119th Meeting, American Chemical Society, p 18M (1951)

[182] D B Cosulich, B Roth, J M Smith, Jr , [illegible] Parker, *J Am Chem Soc* 73, 5006 (1951)

to be blocked by 4-amino PGA under a variety of experimental conditions, while Broquist and co-workers found that this antagonist was reversed

TABLE 8

REVERSAL OF TOXICITY OF FOLIC ACID ANTAGONISTS BY PTEROYLGLUTAMIC ACID (PGA) AND LEUCOVORIN (CF)[179]

Antagonist µg per tube		Growth (optical density) S. faecalis 0.1 µg PGA	L. citrovorum 0.1 mg PGA	L. citrovorum 0.002 µg CF
0		1.22	1.60	1.65
0.03	4-NH_2PGA	0.37	1.43	1.65
0.1		0.20	0.47	1.65
0.3		0	0	1.41
1.0		0	0	0.30
0.1	10-CH_3PGA	0.02	1.60	1.65
1.0		0	1.60	1.65
10		0	1.40	1.65
100		0	0.39	1.68

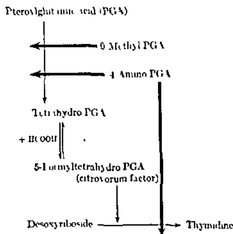


FIG. 3. Possible effects of pteroylglutamic acid antagonists on transformations involving citrovorum factor

competitively by leucovorin (CF) and non-competitively by thymidine in experiments with lactobacilli. Thus, 4-amino PGA is inhibitory against pteroylglutamic acid according to the *square* of the concentration of the antagonist, since it blocks two reactions in the metabolic pathway. Although 10-methyl PGA is strongly inhibitory for *S. faecalis*, this antagonist

systems which would enable to CHO group to be accepted from certain metabolites and transferred to others, and Gordon and co-workers[154] speculated on the role of pteroylglutamic acid in such a system prior to the discovery of the chemical nature of the citrovorum factor. It was suggested by Broquist and co-workers[155] that leucovorin might be reversibly transformed to tetrahydropteroylglutamic acid *in vivo*, in which case tetrahydropteroylglutamic acid should have biological properties similar to those of leucovorin. They found that tetrahydropteroylglutamic acid has indeed a citrovorum-factor-like activity in promoting the growth of *Lc. citrovorum* 8081 and in reversing the toxic effects of Aminopterin for mice

2 Interrelationships of Pteroylglutamic Acid, CF and Pteroylglutamic Acid Antagonists

Interrelationships of pteroylglutamic acid, CF, and the folic acid antagonists in experiments by Nichol and Welch[169] and others have shown that [illegible] into CF

non-competitively in bacterial systems by thymidine. Furthermore, the toxic effects of Aminopterin for mice are reversed competitively by CF but not by pteroylglutamic acid This contrasts with the behavior of "x-methyl pteroylglutamic acid," the effects of which on rats are reversed competitively by pteroylglutamic acid [82]

The folic acid antagonists may be divided into two broad classes based on their behavior towards pteroylglutamic acid. Those in the first class, typified by Aminopterin, show a logarithmic increase in their inhibition indices (ratio of metabolite to antagonist for half-maximum inhibition) with increasing concentration in studies with *S faecalis* The inhibition indices of the second class, typified by 9-methyl PGA, remain approximately constant with increasing concentration of the metabolite Antagonists of the first class are competitively reversed by citrovorum factor and non-competitively reversed by thymidine in studies with *Lc citrovorum*, whereas antagonists of the second class have no effect on this organism, in contrast to *S faecalis*, which appears to be virtually unable to synthesize CF from pteroylglutamic acid These observations, which are illustrated in Table 8, may be reconciled by the scheme presented in Fig 3 The biological conversion of pteroylglutamic acid to CF was shown by Nichol and Welch[169]

[154] M. Gordon, J M Ravel, R E Eakin, and W. Shive, *J Am Chem Soc* **70**, 878 (1948)
[155] H P. Broquist, M J Fahrenbach, J A Brockman, Jr, E L R Stokstad, and T H Jukes, *J Am Chem Soc* **73**, 3535 (1951)
[156] H P Broquist, E L R Stokstad, and T. H Jukes, *J Lab Clin Med* **38**, 95 (1951).

to be blocked by 4-amino PGA under a variety of experimental conditions, while Broquist and co-workers found that this antagonist was reversed

TABLE 8
REVERSAL OF TOXICITY OF FOLIC ACID ANTAGONISTS BY PTEROYLGLUTAMIC ACID (PGA) AND LEUCOVORIN (CF)[170]

	Growth (optical density)		
	S faecalis	*Lc citrovorum*	
Antagonist µg per tube	0 1 µg PGA	0 1 mg PGA	0 002 µg CF
0	1 22	1 60	1 65
0 03 4-NH₂PGA	0 37	1 43	1 65
0 1	0 20	0 47	1 65
0 3	0	0	1 41
1 0	0	0	0 30
0 1 10 CH₃PGA	0 02	1 60	1 65
1 0	0	1 60	1 65
10	0	1 40	1 65
100	0	0 39	1 68

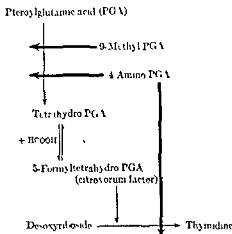


FIG 3 Possible effects of pteroylglutamic acid antagonists on transformations involving citrovorum factor

competitively by leucovorin (CF) and non-competitively by thymidine in experiments with lactobacilli Thus, 4-amino PGA is inhibitory against pteroylglutamic acid according to the *square* of the concentration of the antagonist, since it blocks two reactions in the metabolic pathway. Although 10-methyl PGA is strongly inhibitory for *S. faecalis*, this antagonist

does not block the growth-promoting effect of leucovorin for *Le. citrovorum* This is shown by the results presented in Table 8.

3. The Relationship of Ascorbic Acid to the Conversion of Pteroylglutamic Acid and Citrovorum Factor

Findings in this field, which were discussed on p 360, may throw light on the relation of ascorbic acid deficiency to megaloblastic anemia in infants It has been observed (see p. 330) that this anemia is often associated with ascorbic acid deficiency, although it will not characteristically respond to the administration of vitamin C. It may well be that a deficiency of ascorbic

TABLE 9

Effect of Mode and Administration of Leucovorin on the Urinary Excretion of CF and Folic Acid Activity (FAA) in Adult Men

Subject	Urinary excretion in 6 hr following oral administration of 2.57 mg leucovorin						Urinary excretion in 6 hrs following intravenous injection of 3 05 mg leucovorin		
	Without Ascorbic Acid			1 gm Ascorbic Acid					
	μg CF	% CF recovered	μg FAA	μg CF	% CF recovered	μg FAA	μg CF	% CF recovered	μg FAA
1	28	1 1	50	4	0 16	18	682	22	630
2	39	1 5	63	13	0 5	29	679	22	970
3	32	1 2	66	12	0 5	20	725	24	620
4	31	1 2	51	19	0 7	30			
5	27	1 1	47	32	1 2	36	660	22	540
6	15	0 6	45				652	22	610
Average	29	1 1	54	16	0 6	27	680	22	674

acid accelerates the appearance of the signs and symptoms of folic acid deficiency by diminishing the ability of the tissues to convert pteroylglutamic acid to citrovorum factor Broquist and co-workers[186] noted that ascorbic acid did not increase the amount of citrovorum factor which was excreted in the urine following the administration of a standard dose of leucovorin to normal human subjects. Welch and co-workers[187, 188] have found that the urinary excretion of citrovorum factor in adult human beings with scurvy following a dose of pteroylglutamic acid was found to be very low as compared with that of normal subjects. Infants with megaloblastic anemia respond promptly and completely to pteroylglutamic acid, ad-

[187] A. D Welch, C A Nichol, R M Anker, and J W Boehne, *J. Pharmacol Exptl Therap* 103, 403 (1951)

[188] G J Gabuzda, Jr, G B Phillips, R F Schilling, and C S Davidson, *J Clin Invest* 30, 639 (1951)

ministered orally or parenterally without ascorbic acid, which indicates that pteroylglutamic acid may be utilized to a considerable extent even though the tissues are depleted of ascorbic acid

VII. Pyridoxine Deficiency Anemias

In contrast to the macrocytic anemias which are produced in animals by a deficiency of pteroylglutamic acid, vitamin B_6 deficiency leads to the appearance of a microcytic hypochromic anemia Fouts and co-workers[189, 190] describe a blood dyscrasia in young dogs on a purified diet which occurred in the absence of a source of the rat antidermatitis factor, later identified as pyridoxine The hemoglobin dropped from 12 6 to 15 3 g per 100 ml. of blood to a low value of 2 6 to 3 6 g per cent Simultaneously, the red blood cell count fell to about 30% of its original value, and the mean erythrocyte diameter decreased by about 25%, while the leucocyte count remained normal [190] The administration of either rice bran extract or crystalline pyridoxine was followed by a rise in the reticulocyte count and a rapid disappearance of the microcytic hypochromic anemia Marked reticulocyte responses occurred, which reached peaks on the second or third day of therapy. The mean corpuscular volume in one experiment was 71 to 74 cu μ, which dropped to 48 to 54 cu μ during the deficiency and rose to 62 to 67 cu μ following therapy

Pyridoxine-deficiency anemia in pigs was described by Wintrobe and co-workers [191-194] The anemia was severe, the hemoglobin falling as low as 1.4 g per 100 ml, anisocytosis was marked, and large polychromatic red corpuscles appeared but were outnumbered by the microcytes. The bone marrow was hyperplastic but became normoblastic following therapy As in the case of chicks and dogs, ataxia and convulsions occurred [195] Histological examination of the nervous system showed degeneration occurring in the peripheral nerves, the spinal ganglia, the posterior routes, and the dorsal funiculi of the spinal cord Hemosiderosis of the spleen, liver, and bone marrow was found and could be prevented by restricting the intake of iron although the anemia was aggravated by this procedure Fouts and

[189] P Fouts, O M Helmer, S Lepkovsky, and T H Jukes, *J Nutrition* 16, 197 (1938)

[190] P Fouts, O M Helmer, and S Lepkovsky, *Proc Soc Exptl Biol Med* 40, 4 (1939)

[191] M M Wintrobe, M H Miller, R H Follis, Jr , H J Stein, C. Muschatt, and S Humphreys, *J Nutrition* 24, 345 (1942)

[192] M M Wintrobe, R H Follis, M H Miller, H [illegible]

[193] [illegible]

[194] [illegible] (1944)

[195] E H Hughes and R L. Squibb, *J. Animal Sci* 1, 320 (1942)

co-workers[196] had also noted elevated plasma iron levels in pyridoxine deficiency in dogs Marked and rapid responses were produced in the deficient pigs by the administration of pyridoxine hydrochloride. Intravenous dosage produced greatest responses, but the daily feeding of amounts as low as 80 μg per kilo of body weight was followed by definite and pronounced remissions

Anemia was noted in about one-third of the members of a group of pyridoxine deficient rats by Kornberg and co-workers,[197] and all rats showed an abnormally slow rate of blood regeneration following bleeding

Pyridoxine deficiency in two mentally defective infants was described by Snyderman and co-workers.[198] The infants were kept on a purified deficient diet Pyridoxic acid disappeared from the urine, the infants lost the ability to convert tryptophan to nicotinic acid, and one subject developed a hypochromic anemia at approximately the 130th day. This responded remarkably to pyridoxine; a rise in reticulocytes was noted after 72 hr., reaching a peak in 4 days, after which the red cell count and hemoglobin rose to normal The other subject developed a series of convulsions on the 76th day which necessitated treatment with pyridoxine which was perhaps the reason that anemia was not observed in this second case.

Anemia was not observed by Mueller and Vilter[199] as a result of administering desoxypyridoxine to produce pyridoxine deficiency in adult human subjects.

[196] P Fouts, O M Helmer, and S Lepkovsky, *Am J Med Sci* **199**, 163 (1940)
[197] A Kornberg, H Tabor, and W H Sebrell, Jr, *Am J Physiol.* **143**, 434 (1945)
[198] S E Snyderman, R Carretero, and L E Holt, Jr, *Federation Proc.* **9**, 371 (1950)
[199] J F Mueller and R W Vilter, *J Clin Invest* **29**, 193 (1950)

CHAPTER 11

Vitamin C

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I. Introduction

More laboratory work has been done on vitamin C than on any other vitamin, it is cheap, fairly easy to estimate, and relatively simple in structure and chemical properties, the deficiency disease that it cures has been known in man for centuries and thoroughly investigated But today, twenty years after the first identification of the vitamin with a pure chemical substance, it still cannot be said—apart from its relation to tyrosine—what roles ascorbic acid plays at the molecular level in metabolism

II. Earlier Reviews

Few lengthy and inclusive reviews have been written The chapter by Rosenberg[1] and the monograph of Giroud and Ratsimamanga[2] are the

[1] H. R. Rosenberg, Chemistry and Physiology of the Vitamins, Interscience Publishers, New York, 1942, p 289

[2] A Giroud and A - R Ratsimamanga, Acide ascorbique—Vitamine C, Hermann et Cie, Paris, 1942

best summaries of work done up to 1942 More or less recent reviews[3-8] are useful for different aspects, and more specialized reviews are referred to in the appropriate sections.

III. Chemistry

1. Isolation

Before vitamin C was isolated, scurvy had been known in man for centuries and in the guinea pig for decades[9, 10] Drummond[11] was the first to use the term "water-soluble C" for the antiscorbutic vitamin, and nine years later Szent-Györgyi[12, 13] isolated a so-called "hexuronic" acid from the adrenal cortex In 1932 Tillmans (see, e g , ref 14) suggested that Szent-Györgyi's hexuronic acid was vitamin C, and two independent groups, King and Waugh[15] and Svirbely and Szent-Györgyi,[16] established that hexuronic acid would cure scurvy in the guinea pig The American term "cevitamic acid" has fallen into disuse Szent-Györgyi and Haworth[17] renamed the acid *ascorbic acid* in 1933 Confirmation of the effectiveness of ascorbic acid in curing guinea pig scurvy rapidly appeared[18, 20] One milligram of ascorbic acid is equivalent to 20 International Units of vitamin C.

The synthesis of ascorbic acid made its isolation from natural sources academic Recently paper chromatography[21] has been used to separate it [illegible] [22] have used ion- [illegible] azone of oxidized [illegible]

[3] A P [illegible]
[4] L J [illegible]
[5] W [illegible]
[6] C [illegible]
[7] C G King, *J Am Med Assoc* 142, 563 (1950)
[8] M Pijoan and E L Lozner, *New Engl J Med* 231, 14 (1944).
[9] L J Harris [illegible]
[9] [illegible] 172 (1895–1896).
[10] [illegible]
[11] [illegible]
[12] [illegible]
[13] [illegible]
[14] [illegible]
[15] [illegible]
[16] [illegible]
[17] [illegible]
[18] [illegible]
[20] [illegible]
[21] [illegible]
[22] S [illegible] and C G King, *Arch Biochem Biophys* [illegible] (1951)

I. Introduction

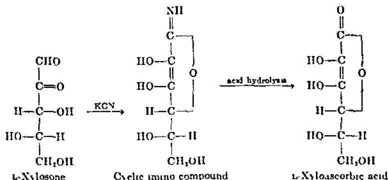
More laboratory work has been done on vitamin C than on any other vitamin, it is cheap, fairly easy to estimate, and relatively simple in structure and chemical properties, the deficiency disease that it cures has been known in man for centuries and thoroughly investigated But today, twenty years after the first identification of the vitamin with a pure chemical substance, it still cannot be said—apart from its relation to tyrosine—what roles ascorbic acid plays at the molecular level in metabolism

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The method has recently been used by Burns and King[33] for the synthesis of 1-C^{14}-L-ascorbic acid Reichstein and Grussner[34] in 1934 evolved the method which is now used in modified form for the commercial synthesis from D-glucose The steps are outlined below The method is of interest, as it seems probable that glucose is the precursor of ascorbic acid *in vivo* (see p 445)

D Glucose —catalytic hydrogenation→ D Sorbitol —acetic acid bacteria→

L-Sorbose —oxidation→ 2 Keto-L-gulonic acid —isomerisation→ L-Xyloascorbic acid

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[33] J J Burns and C G King, *Science* 111, 257 (1950).

[34] T Reichstein and A. Grüssner, *Helv Chim. Acta* 17, 311 (1934)

2. Combined Forms

No convincing evidence had been presented for the existence of a "combined" form of ascorbic acid in living cells until the work of Sumerwell and Sealock was published after the latter's death.[22a] Various workers have proposed (e g , refs. 23 and 24) that a "combined" form, sometimes called ascorbigen, is to be found in plant and animal tissues, and Sumtsov[25] has reviewed this substance Recently other Russian workers, Gol'dshtein, Vol'kenzon, and Kacherova,[26] have claimed that ascorbic acid may be bound with iron and nucleic acids and have given a method for estimating "iron-ascorbic acid" in animal tissues. Sargent and Golden[27] have failed to find bound ascorbic acid in human plasma, in contrast to the conclusion of Schubert [28] The suggestion[29] that ascorbic acid has to be converted to the true vitamin in the organism appears to have gained no further support.

The possibility that the adrenal cortex secretes a compound formed from ascorbic acid and a steroid now seems remote (see p. 413), and it is quite certain that ascorbic acid has so far been found in no naturally occurring compound comparable with cocarboxylase, coenzymes I and II, or visual purple Sumerwell and Sealock[22a] have demonstrated a bound, though readily dissociated, form of ascorbic acid in pig liver The proportion of bound acid rises in scurvy in the guinea pig [29a]

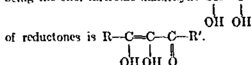
3. Synthesis

Both Rosenberg[1] and Smith[30] give valuable summaries of the methods used for synthesis of the ascorbic acids Reichstein, Grussner, and Oppenauer[31] published their synthesis of L-ascorbic acid just before Haworth's team [32] Both groups used xylosone, and their method is outlined by the scheme given below

[22a] W N Sumerwell and R R Sealock, *J Biol Chem* **196**, 753 (1952)
[23] E Ott, K Krämer, and W Faust, *Z physiol Chem* **243**, 199 (1936)
[24] B Ahmad, *Nature* **136**, 797 (1935)
[25] B M Sumtsov, *Advances in Modern Biol (U S S R)* **27**, 273 (1949) [*C A* **43**, 7107 (1949)]
[26] B I Gol'dshtein, D V Vol'kenzon, and S A Kacherova, *Biokhimiya* **15**, 414 *(1950)*
[27] F Sargent and R Golden, *J Biol. Chem* **188**, 773 (1951)
[28] R Schubert, *Intern Z Vitaminforsch* **19**, 119 (1947)
[29] H Lund, E Trier, M Ottsen, and A Elmby, *Klin Wochschr* **18**, 79 (1939)
[29a] R R. Sealock, R. L Goodland, W. N Sumerwell, and J M Brierly, *J Biol Chem* **196**, 761 (1952)
[30] [illegible]
[31] [illegible], 1019 (1933)
[32] [illegible] Herbert, E [illegible] **1933**, 1419

L-Xyloascorbic acid is colorless, odorless, and acid-tasting, and it crystallizes in plates, m p 192°, $[\alpha]_D^{20}$ + 23° in water

The molecule is almost completely flat[37] One gram dissolves in 3 ml. water, in 50 ml ethanol, and in 100 ml glycerol It is practically insoluble in hydrocarbon solvents[1] Its recrystallization presented considerable difficulties and was effected by Szent-Györgyi[11] from a mixture of methanol, ether, and petroleum ether The ene-diol structure of carbon atoms 2 and 3 permits its classification as a reductone, the simplest member of this class being the enol tartronic dialdehyde CH═C—CHO, the general structure



of reductones is R—C═C—C—R′.

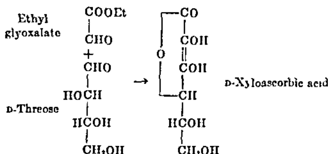
The ene-diol grouping gives ascorbic acid two of its most characteristic properties First, though a lactone of a carboxylic acid, it is a dibasic acid (pK_1 = 4 17 and pK_2 = 11 57);[38] and second, it is very readily oxidized There have been difficulties in measuring electrode potentials of the system, E_0' being +0 051V at pH 7 24 and 30° in water[39, 39a] Dixon's rH diagram gives a valuable comparison with other systems[40] The ultimate product of mild oxidation has been known as "dehydroascorbic acid," but it has no acidic properties and is perhaps better named "ascorbone" in view of its ketonic and antiscorbutic properties Ascorbic acid and ascorbone are thus analogous with the hydrojuglone and juglone of walnuts, for example The salts of ascorbic acid are generally very soluble in water, and the relative insolubility of the basic lead salt has been used for isolation and purification

The reaction between ascorbone and amino acids has been examined by Abderhalden[41] and Moubasher,[42] who showed that ascorbone and phenylglycine react together under physiological conditions to give benzaldehyde. The decarboxylation of ascorbone formed by the action of ninhydrin on ascorbic acid has been observed[42a]

Drake, Smythe, and King[43] have provided physical and chemical evi-

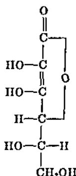
37 E G Cox and T H, Goodwin, *J. Chem Soc* 1936, 769
38 T W Birch and L. J Harris, *Biochem J* 27, 595 (1933)
39 E G Ball, *J Biol Chem* 118, 219 (1937)
39a L F Hewitt, Oxidation-Reduction Potentials in Bacteriology and Biochemistry, 6th ed , Livingstone, Edinburgh, 1950, p 60
40 M Dixon, Multi-enzyme Systems, Cambridge University Press, Cambridge, 1949, p 73
41 E [illegible]
42 R [illegible]
42a E [illegible] 42).
43 B [illegible] 143, 89 (1942)

The method of Helferich and Peters,[25] whereby a C_2 and a C_4 derivative are condensed to give D-ascorbic acid, is of interest in relation to the probable breakdown of dehydroascorbic acid *in vivo* into oxalic acid and a C_4 derivative (see p 446), though *in vitro* L-ascorbic acid has not been made in quantity thus because of the rarity of the C_4 starting material. An outline of the process for D-ascorbic acid is given below.



4 Properties

The chemistry of ascorbic acid was partly worked out by Herbert *et al.*[26] on samples isolated by Szent-Györgyi before a synthesis had been effected They showed that L-ascorbic acid has the structure given below.



The terminology based on the starting sugar used in the osone-cyanohydrin synthesis is a useful one for naming the various isomers and homologues that have been synthesized and used in chemical and biological work Ascorbic acid is thus known as L-xyloascorbic acid, and "isoascorbic" acid, which may be readily prepared by oxidation of fructose, is best known as D-araboascorbic acid, as it may also be obtained from the cyanohydrin of D-arabosone.

[25] B Helferich and O Peters, *Ber* 70, 465 (1937)

[26] R. W. Herbert, E L. Hirst, E. G V Percival, R J W Reynolds, and F Smith, *J Chem Soc.* 1933, 1270.

higher than that of indophenol, and the method is potentially some six or ten times more sensitive For complete reduction in dilute solution the mixture has to be strongly irradiated with red light. The aerobic oxidation of leucomethylene blue proceeds rapidly, and this reoxidation seems to have vitiated some of the results of earlier workers, who claimed that the lower results for ascorbic acid obtained with methylene blue demonstrated its greater specificity [51] The best investigation of the reaction, which is of general interest, was published by Straub,[52] and Wahren,[53] using a low intensity of radiation, obtained evidence that methylene blue first oxidizes ascorbic acid to an intermediate form, possibly monodehydroascorbic acid, before ascorbone itself is produced Recent work on micro methods using methylene blue has been done by Raoul[54] and de Gandarias [55]

There are several other methods depending on oxidation· in that of El Ridi, Moubasher, and Hassan,[56] ascorbic acid reduces colorless perinaphthindantrione hydrate to a red reduction product, so that the color developed is proportional to the ascorbic acid originally present In the indophenol colorimetric method the ascorbic acid is proportional to the reduction in color

The method of Roe and Kuether[57] measures the sum of ascorbic acid, ascorbone, and diketogulonic acid as a bis-2,4-dinitrophenylhydrazone of the latter, in a fairly concentrated aqueous solution of sulfuric acid this gives an intense red color The method is sensitive, accurate, and precise, and, although not all ascorbic acid in living tissues or food is present in the reduced form, most of it usually is Before estimation, therefore, it is oxidized, and thus any losses of reducing power during extraction are immaterial The method is excellent for estimating ascorbic acid in whole blood or bloody tissues, as precipitation of oxyhemoglobin is generally accepted as causing complete oxidation of ascorbic acid (see p 385; contrast ref 58) It has been adapted for separately estimating ascorbic acid, ascorbone, and diketogulonic acid,[59] and as a micro method by Bessey, Lowry, and Brock,[60] particularly for application to leucocytes from capillary blood

The estimation of ascorbic acid by means of its opaque band in the

[51] H Lund and H Lieck, *Klin Wochschr* 16, 555 (1937)
[52] F B Straub, *Z physiol Chem* 254, 147 (1938)
[53] H Wahren, *Klin Wochschr* 16, 1496 (1937)
[54] Y Raoul, *Bull soc chim biol* 29, 728 (1947)
[55] J M de Gandarias, *Rev españ. fisiol* 7, 81 (1951)
[56] M S El Ridi, R Moubasher, and Z F. Hassan, *Biochem J.* 49, 246 (1951).
[57] J H Roe and C A Kuether, *J Biol Chem.* 147, 399 (1943)
[58] D J Stephens and E E Hawley, *J Biol Chem* 115, 653 (1936)
[59] J H Roe, M B Mills, M J Oesterling, and C M Damron, *J Biol. Chem* 174, 201 (1948).
[60] O A Bessey, O H Lowry, and M J Brock, *J Biol. Chem* 168, 197 (1947)

dence for the existence of addition compounds between one molecule of ascorbone and one molecule of thiols such as glutathione.

A third characteristic property resulting from the ene-diol grouping is the intense absorption band at 245 mμ in acid aqueous solution, moving to 265 mμ in neutral solution These peaks have no counterpart in ascorbone.[1] Weigl[43a] has recently shown from a study of the infrared spectrum of deuterated ascorbic acid that there is one labile hydrogen atom attached to carbon, possibly C_4

The production of ascorbone is of importance. Charcoal,[44] benzoquinone,[43] and iodine[45] have been used for oxidizing ascorbic acid Herbert *et al*[35] supposed that the first product of oxidation was a tetrahydroxyl form made by the addition of two hydroxyl groups to the double bond, and it is possible that different methods of oxidation may result in products differing with respect to the hydration of the carbonyl groups (see also ref. 46).

Most of the physical and chemical properties of ascorbic acid that are known to be important for the physiologist have been mentioned. Further properties emerge in the course of the discussion in later sections

5. Estimation

Tillmans, Hirsch, and Reinshagen[47] made an important advance in the study of vitamin C when they used the dye 2,6-dichlorophenolindophenol for titrating meat juice, milk, and lemon juice Szent-Gyorgyi used iodine for following hexuronic acid during its isolation, but dye titration was the first widely used method for estimating ascorbic acid after its isolation and synthesis The titration is best done in acid solution, buffered to about pH 4 0; at this pH the reduction of the dye is almost complete and therefore stoichiometric, the excess dye does not fade as rapidly as in more acid solutions, and interference from some other reducing substances is relatively slight [48] The indophenol method has been very widely used in an impressive array of modifications, and its sensitivity and simplicity make it the ideal method for certain types of work The kinetics of the reaction have been examined by Hochberg, Melnick, and Oser.[49]

Martini and Bonsignore[50] recommended the replacement of indophenol with methylene blue The molar absorptivity of methylene blue is much

[43a] J. W Weigl, *Anal Chem* **24**, 1483 (1952).
[44] F W. Fox and L. F Levy, *Biochem J.* **30**, 208 (1936)
[45] J. Kenyon and N Munro, *J Chem Soc.* **1948**, 158
[46] B. Pecherer, *J. Am Chem Soc* **73**, 3827 (1951)
[47] J. Tillmans, P. Hirsch, and E Reinshagen, *Z. Untersuch Lebensm* **56**, 272 (1928).
[48] R L. Mindlin and A. M Butler, *J. Biol Chem.* **122**, 673 (1938).
[49] M Hochberg, D Melnick, and B. L. Oser, *Ind Eng Chem , Anal Ed* **15**, 182 (1943)
[50] E. Martini and A Bonsignore, *Biochem Z* **273**, 170 (1934)

TABLE I

The Antiscorbutic Activity of Various Compounds

Compound	Activity	Reference
L-Xyloascorbic acid	100	5
D-Araboascorbic acid	5	5
D-Xyloascorbic acid	0	5
D-Glucoascorbic acid	0	5
	Some?	65a
D-Galactoascorbic acid	Some?	65b
L-Rhamnoascorbic acid	20	5
L-Araboascorbic acid	0	65b
L-Glucoascorbic acid	2 5	5
L-Galactoascorbic acid	1 0	5
6-Desoxy-L-ascorbic acid	33	1
L-Fucoascorbic acid	2	1
D-Glucoheptoascorbic acid	1?	65b
L-Scorbamic acid	Some	1
3 Methyl-L-ascorbic acid	ca. 2–4	66
Methyl 2-keto-L-gulonate	ca 40	1, 67

7 Occurrence

L-Xyloascorbic acid, so far the only ascorbic acid to be found occurring in nature (though see p 441), is widespread throughout the plant and animal kingdoms It is not found in most unicellular organisms, and of the mammals so far examined only the primates, the guinea pig, and the marmot have been shown to need it in their diets The concentration of ascorbic acid in the parts of the animal body varies widely (see later sections), and its concentration in plants varies with functional activity and season With reference to the human diet, any food containing 10 mg of ascorbic acid in the quantity normally eaten at a meal is a valuable source The potato is probably the most important single source in northwestern Europe

IV. Metabolism in Animals

1 Distribution and Dynamics

a. Organs and Tissues. The distribution of vitamin C in animals involves two concepts, its distribution in the various parts of the organism, and its state of oxidation, i e , the proportion of it present as ascorbone

Penney and Zilva[70] have shown that in all tissues of the guinea pig except peritoneal leucocytes the total ascorbic acid content decreases exponentially with time when the animal is on a deficient diet Apart

[70] J R Penney and S S Zilva, *Biochem J*, 40, 695 (1946).

ultraviolet has been recently described by Vacher and Faucquembergue[61] and Daglish[62]

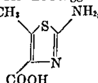
The powerful methods described above for estimating ascorbic acid and its products in biological media have greatly assisted the assessment of the vitamin C content of foodstuffs and of the C nutriture of human beings. The slowness of the advance in fundamental knowledge of vitamin C cannot be laid at the door of the chemists who have developed these methods, unless it could be argued that too much time has been transferred from application to development. Up-to-date accounts of various methods are to be found in "Methods of Vitamin Assay"[63] and György.[64] Histochemical methods, based on the reduction of silver nitrate, have recently been reviewed by Glick[65]

6. Substitutes

Soon after the first synthesis of L-xyloascorbic acid, some of its homologues, isomers, and derivatives were prepared and tested for their antiscorbutic properties in the guinea pig Table 1, compiled mainly from data in Eddy[5] and Rosenberg,[1] expresses the relative activities of various compounds

It appears that for antiscorbutic activity the D configuration is necessary at carbon atom 4, with the doubtful exceptions of D-glucoascorbic acid and D-galactoascorbic acid The presence of a labile C—H bond in ascorbic acid[43a] raises the question of the interconversion of the various optical isomers It has been suggested by Zilva[68] that activity of some compounds may be related to their retention by the kidney.

The activity of methyl nornarcotine is discussed on p 449 Di Maggio[69] claims that 2-amino-5-methylthiazole-4-carboxylic acid,



partly protects guinea pigs against scurvy.

[61] M. Vacher and D Faucquembergue, *Bull soc chim biol* 31, 1419 (1949)
[62] C. Daglish, *Biochem J* 49, 635 (1951)
[63] Association of Vitamin Chemists, Inc., Methods of Vitamin Assay, 2nd ed , Interscience Publishers, New York, 1951
[64] P. György (Ed), Vitamin Methods, Academic Press, New York, 1951, Vol 2.
[65] D Glick, Techniques of Histo- and Cytochemistry, Interscience Publishers, New York, 1949
[65a] W. G. Shafer, *J Dental Research* 29, 831 (1950)
[65b] T. Reichstein and V Demole, Festschrift für E C. Barell, Basel, 1936
[66] [illegible]
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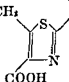
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[illegible] *biol* 31, 1419 (1949)
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[illegible] of Vitamin Assay, 2nd ed ,
[illegible] s, New York, 1951, Vol 2.
[illegible] y, Interscience Publishers, New York, *1949*
[illegible] (1950)
[illegible] 36
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D-Glucoheptoascorbic acid	1?	65b
L-Scorbamic acid	Some	1
3-Methyl-L-ascorbic acid	ca 2–4	66
Methyl 2-keto-L-gulonate	ca 40	1, 67

7 Occurrence

L-Xyloascorbic acid, so far the only ascorbic acid to be found occurring in nature (though see p 441), is widespread throughout the plant and animal kingdoms It is not found in most unicellular organisms, and of the mammals so far examined only the primates, the guinea pig, and the marmot have been shown to need it in their diets The concentration of ascorbic acid in the parts of the animal body varies widely (see later sections), and its concentration in plants varies with functional activity and season With reference to the human diet, any food containing 10 mg of ascorbic acid in the quantity normally eaten at a meal is a valuable source The potato is probably the most important single source in northwestern Europe

IV. Metabolism in Animals

1 Distribution and Dynamics

a. Organs and Tissues. The distribution of vitamin C in animals involves two concepts, its distribution in the various parts of the organism, and its state of oxidation, i e , the proportion of it present as ascorbone.

Penney and Zilva[70] have shown that in all tissues of the guinea pig except peritoneal leucocytes the total ascorbic acid content decreases exponentially with time when the animal is on a deficient diet Apart

[70] J R Penney and S S Zilva, *Biochem J.* 40, 695 (1946).

TABLE 2

THE DISTRIBUTION OF ASCORBIC ACID IN THE GUINEA PIG ON VARIOUS INTAKES OF VITAMIN C

Intake	Blood	Liver	Kidney	Adrenal	Spleen	Stomach	Small intestine	Large intestine	Muscle	Marrow	Brain	Heart	Ref
	mg /100 ml.	mg /100 g											
Mixed cabbage *ad lib*	0 75	16 7	8 5	150	43	11 0	20 4	10 3	3 1				70
Cabbage	0 75	23 8	9 1	119	43				2 39		18 6	7 5	71
"Saturation"	0 25	9 7	5 5	96	34	7 5	14 0	8 5	2 3				70
Deficient diet, 0 5 mg orally/day	0 10	1 1	0 82	10	3 8	0 84	1 8	1 1	0 44	2 1			70
0 18 mg /100 g /day	0 09	0 79	0 55	4 52	2 25				0 20		3 05	0 29	71
2 0 mg /day	0 13	1 7	1 3	21	9 3	1 7	4 2	2 2	0 44	3 2			70
0 70 mg /100 g /day	0 14	3 75	2 48	28 5	12 8				0 76		8 35	2 16	71
6 0 mg /day	0 14	3 3	2 3	32	11	3 0	5 9	3 8	1 0	6 6			70
1 83 mg /100 g /day	0 35	11 6	5 64	73 9	26 0				1 63		15 2	4 59	71
12 0 mg /day	0 20	5 2	2 9	34	16	3 9	6 5	4.1	1 0	6 9			70
3 44 mg /100 g /day	0 54	15 5	7 87	110 5	33 0				2 24		18 4	5 49	71
25 0 mg /day	0 22	6 1	5 2	65	25	5 7	10 6	6 1	1 3	8 9			70

from blood and muscle, the other tissues examined showed a tenfold reduction in about 13 days Blood showed a two- to threefold reduction in the same period, and muscle was intermediate between blood and the other tissues.

Table 2, giving the distribution of ascorbic acid in guinea pigs on graded intakes, is compiled from Penney and Zilva[70] and Kuether, Telford, and Roe[71] For tissues other than blood, muscle, heart, and leucocytes (not in

TABLE 3

THE DISTRIBUTION OF ASCORBIC ACID IN VARIOUS MAMMALS*

	Ox (2)†	Horse (2)	Dog (2)	Sheep (2)	Rat (2)	Guinea pig		Man	
						Cabbage (70, 71)	Ca 2 mg/day (70, 71)	(72)	(73)
Brain	16 6	18 5	13 4	15 4	27	18 6	8 4	11–46	6 2
Hypophysis	126	136	101	139 6	106				
Testicle	30	46	45	34	26 3				3 1
Thyroid	17	18	16 5	31 7	22				2 1
Stomach	6 3	8	4	6 5	16 6	11 0	1 7		2 8
Small intestine	18	17	18	20 2	22 6	20 4	4 2		2 0
Large intestine	7 3	6 8	7	10 4	19	10 3	2 2		1 3
Lymphatic ganglion	51	44	27 6	45 4	57				
Lung	18 2	18	14 6	12 6	27			4–13	1 0
Skeletal muscle	1 6	1 3	1 7	2 55	3 1	3 1	0 4		
Cardiac muscle	3 8	3 3	3 6	6 2	4 6	7 5	2 2	2–8	
Smooth muscle	6 3	5 3	4	10 8	18 7				
Liver	20–37					16 7	1 7	6–16	5 0
Spleen	27 5	29	24 4	34	32 6	43	9 3	8–16	30 0
Lymph									
Kidney						8 5	1 3	5–15	4 3
Adrenal	97-160					150	21	25–38	15 5

* Milligrams per 100 g

† Numbers in parentheses are reference numbers

the table), the concentration varies directly and fairly exactly with the square root of the daily intake

Table 3 gives concentrations of ascorbic acid in milligrams per 100 g in tissues from various mammals.

The degree of oxidation of ascorbic acid in the tissues has been investigated by Martini and Bonsignore,[74] by Banerjee, Deb, and Belavady,[75]

[71] C. A Kuether, I R Telford, and J H Roe, *J Nutrition* 28, 347 (1944)

[72] M Yavorsky, P. Almaden, and C G King, *J Biol Chem* 106, 525 (1934).

[73] E Góth and I Littmann, *Orvosok Lapja Népegészségügy* 3, 518 (1947)

[74] E Martini and A. Bonsignore, *Boll soc ital biol sper* 9, 1240 (1934)

[75] S Banerjee, C Deb, and B Belavady, *J Biol Chem* 195, 271 (1952)

TABLE 2

THE DISTRIBUTION OF ASCORBIC ACID IN THE GUINEA PIG ON VARIOUS INTAKES OF VITAMIN C

Intake	Blood	Liver	Kidney	Adrenal	Spleen	Stomach	Small intestine	Large intestine	Muscle	Marrow	Brain	Heart	Ref
	mg /100 ml	mg /100 g											
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Cabbage	0 75	23 8	9 1	119	43				2 39		18 6	7 5	71
"Saturation"	0 25	9 7	5 5	96	34	7 5	14 0	8 5	2 3				70
Deficient diet, 0 5 mg orally/day	0 10	1 1	0 82	10	3 8	0 84	1 8	1 1	0 44	2 1			70
0 18 mg /100 g /day	0 09	0 79	0 55	4 52	2 25				0 20		3 05	0 29	71
2 0 mg /day	0 13	1 7	1 3	21	9 3	1 7	4 2	2 2	0 44	3 2			70
0 70 mg /100 g /day	0 14	3 75	2 48	28 5	12 8				0 76		8 35	2 16	71
6 0 mg /day	0 14	3 3	2 3	32	11	3 0	5 9	3 8	1 0	6 6			70
1 83 mg /100 g /day	0 35	11 6	5 64	73 9	26 0				1 63		15 2	4 59	71
12 0 mg /day	0 20	5 2	2 9	34	16	3 9	6 5	4 1	1 0	6 9			70
3 44 mg /100 g /day	0 54	15 5	7 87	110 5	33 0				2 24		18 4	5 49	71
25 0 mg /day	0 22	6 1	5 2	65	25	5 7	10 6	6 1	1 3	8 9			70

from blood and muscle, the other tissues examined showed a tenfold reduction in about 13 days Blood showed a two- to threefold reduction in the same period, and muscle was intermediate between blood and the other tissues

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Hypophysis	126	136	101	139 6	106				
Testicle	30	46	45	34	26 3				3 1
Thyroid	17	18	16 5	31 7	22				2 1
Stomach	6 3	8	4	6 5	16 6	11 0	1 7		2 8
Small intestine	18	17	18	20 2	22 6	20 4	4 2		2 0
Large intestine	7 3	6 8	7	10 4	19	10 3	2 2		1 3
Lymphatic ganglion	51	44	27 6	45 4	57				
Lung	18 2	18	14 6	12 6	27			4–13	1 0
Skeletal muscle	1 6	1 3	1 7	2 55	3 1	3 1	0 4		
Cardiac muscle	3 8	3 3	3 6	6 2	4 6	7 5	2 2	2–8	
Smooth muscle	6 3	5 3	4	10 8	18 7				
Liver	20–37					16 7	1 7	6–16	5 0
Spleen	27 5	29	24 4	34	32 6	43	9 3	8–16	30 0
Lymph									
Kidney						8 5	1 3	5–15	4 3
Adrenal	97–160					150	21	25–38	15 5

* Milligrams per 100 g

† Numbers in parentheses are reference numbers

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The degree of oxidation of ascorbic acid in the tissues has been investigated by Martini and Bonsignore,[74] by Banerjee, Deb, and Belavady,[75]

[71] C A Kuether, I R Telford, and J H Roe, *J Nutrition* 28, 347 (1944)

[72] M Yavorsky, P Almaden, and C G King, *J Biol Chem* 106, 525 (1934)

[73] E Góth and I Littmann, *Orvosok Lapja Népegészségügy* 3, 518 (1947)

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[75] S Banerjee, C Deb, and B Belavady, *J Biol Chem* 195, 271 (1952)

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Deficient diet, 0 5 mg orally/day	0 10	1 1	0 82	10	3 8	0 84	1 8	1 1	0 44	2 1			70
0 18 mg /100 g /day	0 09	0 79	0 55	4 52	2 25				0 20		3 05	0 29	71
2 0 mg /day	0 13	1 7	1 3	21	9 3	1 7	4 2	2 2	0 44	3 2			70
0 70 mg /100 g /day	0 14	3 75	2 48	28 5	12 8				0 76		8 35	2 16	71
6 0 mg /day	0 14	3 3	2 3	32	11	3 0	5 9	3.8	1 0	6 6			70
1 83 mg /100 g /day	0 35	11 6	5 64	73 9	26 0				1 63		15.2	4 59	71
12 0 mg /day	0 20	5 2	2 9	34	16	3 9	6 5	4 1	1 0	6 9			70
3 44 mg /100 g /day	0 54	15 5	7 87	110 5	33 0				2 24		18.4	5 49	71
25 0 mg /day	0 22	6 1	5 2	65	25	5 7	10 6	6 1	1 3	8 9			70

result of a secretory process, with Jackson[79] he found that in the monkey at birth the aqueous and plasma concentrations were equal, and after the first month the aqueous concentration rose until the adult level of six to eight times that of the plasma was reached at about 6 months In man the normal adult aqueous level is probably reached during the last 2 months of fetal life.

Langham[80] has shown that in the rabbit, though not in the cat, intravenous ascorbone causes a greater elevation of ascorbic acid in the aqueous than does ascorbic acid itself, and he suggests that the accumulation of ascorbic acid in the aqueous involves a reduction of ascorbone by the lens and other intraocular tissues (cf ref 81) Langham's results seem to provide a partial explanation of Kinsey's observations though they do not identify the normal source of ascorbone from which aqueous ascorbic acid may be formed by reduction. they may be compared with Lloyd's[82] results on the human erythrocyte *in vitro* and Patterson and Mastin's[83] results on the nervous system of the rat *in vivo* (see pp 387 and 390)

The speculation that the ascorbic acid-ascorbone system acts as a source of oxygen for the metabolism of certain ocular structures which are sparsely supplied with blood is attractive but as yet unsupported by direct experimental evidence, and no striking changes in visual function or ocular structure have been noticed in clinical scurvy Campbell and Ferguson[84] have shown that the heat-injured cornea shows vascularization more frequently in the scorbutic than in the normal guinea pig. Their controls were not pair fed, however

It appears that ascorbic acid has a general effect on the blood-aqueous barrier Simonelli[85] has shown that intravenous injection of 0 5 to 1 0 g into human subjects causes its increased permeability in normal and diseased eyes In apparent contrast Morone and Nižetić[87] have shown in guinea pigs on a scorbutic diet that the barrier's permeability advances with deficiency

(2) *Blood* The study of ascorbic acid and ascorbone in blood has been undertaken, since 1933, by a large number of workers, and some of the complexities of the relations are now being appreciated

The comparative ease of estimating reduced ascorbic acid in plasma and the belief that this estimation reveals the vitamin C nutriture of the donor have resulted in the publication of a very large number of results

and by Damron, Monier, and Roe[76] after administration of ascorbic acid, ascorbone, and diketogulonic acid

It is now generally agreed that ascorbic acid is usually almost entirely in the reduced form in the tissues but that in scurvy the ratio of ascorbone to ascorbic acid rises This ratio is sometimes known as the "scorbutic index," and it is pointed out elsewhere (p 413) that the rising of the index in scurvy may be regarded as a maintenance of the product of the concentrations of ascorbic acid and ascorbone Estimations of the index in resting and "stressed" adrenal cortices do not seem to have been made

[illegible]

Damron *et al* used hydrazine methods Although ascorbone prevents

TABLE 4

ASCORBONE AND ASCORBIC ACID (MG /100 G) IN ORGANS OF SCORBUTIC (S) AND NORMAL (N) GUINEA PIGS, MEAN VALUES

Tissue	Whole animal		Adrenals		Brain		Pancreas		Spleen		Blood		Intestine		Muscle		Kidney		Liver		Reference
	N	S	N	S	N	S	N	S	N	S	N	S	N	S	N	S	N	S	N	S	
Ascorbone																					75
																					76
Ascorbic acid																					75
																					76

*Gastrointestinal tract

scurvy, it allows only very little storage of ascorbic acid in the tissues[77, 77a] (cf. p 415) No determination of the period of deprivation needed to establish scurvy after an ascorbone diet seems to have been made (cf. p 464 and ref. 70)

(1) *Eye* The investigation of the relation between ascorbic acid and the eye has received impetus in recent years.[77b] Four years after the important paper of Friedenwald, Buschke, and Michel,[77c] Kinsey[78] showed that L-xyloascorbic acid and D-araboascorbic acid, but not D-glucoascorbic acid, enter the aqueous humor of the eye of the adult albino rabbit as a

[76] C M Damron, M M Monier, and J H. Roe, *J. Biol Chem* 195, 599 (1952).
[76a] E Martini and F Pinotti, *Boll soc ital. biol sper* 10, 58 (1935).
[77] [illegible]
[77a] [illegible]
[77b] [illegible] *'hicago)*
[77c] [illegible] 29, 535 (1943)
[78] V E. Kinsey, *Am J Ophthalmol.* 30, 1263 [illegible]

Robson[94] have used the indophenol and hydrazine methods, with careful control of their techniques, to show that some 20% of human plasma ascorbic acid is present in the oxidized form (cf ref 94a) The effects of cortisone and corticotropin on this proportion are discussed on p 416, and the relation of ascorbone to the erythrocytes later in this section It was earlier thought (e g , ref 95) that all vitamin C in blood is present as ascorbone, but during protein precipitation reduced ascorbic acid in blood is oxidized, probably by the oxygen leaving oxyhemoglobin during its de-

TABLE 5

ASCORBIC ACID IN HUMAN DIET AND SERUM

Dietary intake in mg ascorbic acid per day	Number of subjects	Serum ascorbic acid in mg /100 ml
<15	18	0 05–0 15
15–30	26	0 10–0 39
30–50	48	0 11–0 85
50–100	47	0 51–0 95
>100	75	0 70–1 94

TABLE 6

ASCORBIC ACID IN HUMAN DIET, SERUM, AND WHITE LAYER

Group	Ascorbic acid, mg / day	Serum ascorbic acid, mg /100 ml			White layer ascorbic acid, mg /100 g		
		Lowest in group	Mean	Highest in group	Lowest	Mean	Highest
1	8 (basal diet)	0 03	0 18	0 4	4	11 9	19
2	23 (1 + cabbage)	0 06	0 20	0 3	7	12 9	19
3	78 (1 + 70 mg synthetic)	0 15	0 79	1 7	20	24 2	33
4	78 (normal rations)	0 3	0 73	1 6	15	23 7	32

naturation,[96] and it seems probable that in most tissues precipitated in the presence of hemoglobin from which oxygen has not been rigorously excluded (cf ref 97) considerable [illegible]

[94] C P Stewart, B D Horn, and J S Robson, *Biochem J* 53, 254 (1953)

[94a] B B Lloyd, H M Sinclair, and G R Webster, *Biochem J* 39, xvii (1945)

[95] M van Eekelen, A Emmerie, B Josephy, and L K Wolff, *Nature* 132, 315 (1933)

[96] A E Kellie and S S Zilva, *Biochem J* 30, 361 (1936)

[97] A Fujita, F Ebihara, and I Numata, *Biochem Z* 301, 215 (1939)

for ascorbic acid in human plasma or serum There is undoubtedly a correlation between plasma ascorbic acid and dietary intake, but for the individual member of a surveyed population this correlation may be of little value in interpreting a plasma level Genuine zero values are rare They are sometimes found in scurvy and sometimes before scurvy has appeared On p. 397 the work demonstrating that ascorbic acid is a threshold substance is discussed: the renal tubular maximum for reabsorption of ascorbic acid means that levels above 1 5 mg. per 100 ml are very rare in the resting post-absorptive subject. The relationship, in man, between intake and serum level is summarized in Table 5 from the data of Odin [89]

These figures may be compared with those for serum ascorbic acid given by Lowry *et al* [90] They examined 100 members of the Royal Canadian Air Force, divided into four groups, and kept for 8 months on diets of known ascorbic acid contents; their results for ascorbic acid in serum and the white layer are given in Table 6

The overlap of values between the first and second pairs of groups is striking The differences between groups 1 and 2 and between groups 3 and 4 were not statistically significant, those between the first and second pairs of groups were significant.

Fox and Dangerfield,[91] in an important study on indigenous mineworkers in South Africa, gave a group of about 1000 a supplement of about 40 mg per day and used a second group of about 1000 as a control group After 6 months, plasma estimations on samples of about 80 men from the two groups showed only a very slight mean difference of about 0 02 mg per 100 ml, the supplemented group yielding the higher mean of 0 25 mg per 100 ml There were 12 cases of scurvy in the control group and 1 doubtful case in the experimental group Borsook *et al* [92] found that a supplement of 250 mg five times a week caused very considerable increases in mean plasma levels in American factory workers, and it is just possible that Fox's apparently anomalous results are due to a racial difference Fox's low figures contrast with the extremely high figures, going with apparently low dietary intakes, found in Mexican Indians,[93] the dietary estimates possibly omitted very important "native" sources of ascorbic acid in the latter study

Results for ascorbone in plasma are not abundant Stewart, Horn, and

[89] M Odin, *Acta Paediat* **26**, 339 (1939)
[90] O H Lowry, O A Bessey, M J Brock, and J A Lopez, *J Biol Chem* **166**, 111 (1946)
[91] F W Fox and L F. Dangerfield, *Proc. Transvaal Mine Med Officers Assoc* **19**, 267 (1940)
[92] H Borsook, J W. Dubnoff, G Keighley, and D G Wiehl, *Milbank Mem Fund Quart* **24**, 99 (1946)
[93] P. Putnam, D. F. Milam, R K. Anderson, W J
bank Mem Fund Quart **27**, 355 (1949)

blood and to various components of defibrinated blood and concluded that erythrocytes prevented the loss of ascorbic acid in serum exposed to air, and that leucocytes plus erythrocytes hastened it Heinemann also found that the movement of added ascorbic acid from serum to cells as a whole depended on the number of leucocytes present For technical reasons he did not do separate estimations in erythrocytes and leucocytes, and his final conclusions were that ascorbic acid *in vitro* passes to leucocytes only and not to erythrocytes Another explanation of his results is that the presence of leucocytes causes the conversion of ascorbic acid to a compound that enters the erythrocyte, and Lloyd[82] has shown that in air ascorbic acid added to defibrinated human blood passes to the erythrocytes at a rate which increases with an increase in the quantity of leucocytes present, ascorbone itself (prepared with charcoal) passes very quickly, in the absence or presence of leucocytes, to the erythrocytes, where it is largely reduced to ascorbic acid (see also p 424) The addition of ascorbone, but not of ascorbic acid, causes an increased accumulation of total ascorbic acid in the leucocytes, though the rate of accumulation is much slower than in erythrocytes The removal of the erythrocytes allows the leucocytes to accumulate ascorbic acid after the addition of ascorbic acid,[108] and it seems that, whereas the human erythrocyte *in vitro* rapidly acquires ascorbic acid by reduction of surrounding ascorbone, rather as do the nervous system of the rat[83] and the aqueous of the rabbit,[81] the leucocytes' level of ascorbic acid may be controlled by the surrounding concentration of ascorbone or by its ratio to reduced ascorbic acid in the surrounding medium This may explain the high ascorbate levels found after incubation *in vitro* in leucocytes in normal defibrinated blood deprived of its erythrocytes,[108] and *in vivo* in some leukemic bloods, for in these systems the mechanism of removal of ascorbone by reduction in the erythrocyte is absent or diminished and its steady-state concentration presumably tends to be raised to a new high level The hydrazine method of estimation shows that considerable conversion of ascorbic acid to substances other than diketogulonic acid occurs when it is incubated with human whole blood; breakdown to oxalic acid seems likely.

The high concentration of ascorbic acid in the leucocytes and the availability of these living cells from normal human subjects and patients and

[108] B. B Lloyd, unpublished data

The response of plasma ascorbic acid to large intravenous or oral doses varies with the previous vitamin C nutrition, and the rise and fall of the plasma concentration is in accordance with the existence of a renal threshold The erythrocyte concentration lags behind that of plasma The diagnostic advantages of the test are probably not greater than urinary load tests or direct single analyses on the components of blood without dosing (cf ref 98).

The concentration of vitamin C in the erythrocyte is usually very similar to that in the plasma, especially when due allowance is made for water content [58, 100, 101] The concentration of ascorbic acid in the leucocytes and platelets, or white layer, of blood is very much higher than in the plasma or erythrocytes, rising to about 40 mg per 100 g. in normal subjects and to 139 mg per 100 g in a case of lymphatic leukemia after dosage with vitamin C Stephens and Hawley[58] and Cuttle[102] were the first to suggest that ascorbic acid accumulated in the leucocytes, that the reducing substance present was ascorbic acid was virtually proved by Butler and Cushman,[103] who isolated the bishydrazone formed from the compound in leukemic leucocytes and showed that its properties were very close to the bishydrazone from pure ascorbic acid and that the two bishydrazones showed no lowering of melting point on mixing.

Crandon, Lund, and Dill[104] in their classical deficiency experiment showed that the white layer lost ascorbic acid very much more slowly than did the plasma, which became devoid of ascorbic acid after 41 days, with no demonstrable clinical signs After 103 days the white layer ascorbic acid became zero, and 31 days after this follicular hyperkeratosis appeared. Subsequent experiments have shown that the white layer ascorbic acid is a better index of nearness to scurvy than is the plasma level, though the correlation between dietary intake and level is not very much greater than with plasma (see Table 6) Lubschez[101] found values up to 58 mg per 100 g in the white layer of children, but they were not closely correlated with the fasting plasma level In the guinea pig the leucocytes retain ascorbic acid at a fairly constant but low level (ca 2 mg per 100 g) during the later stages of total deprivation [70]

The "dynamics" and metabolism of vitamin C in human blood have been investigated relatively little. Heinemann (e g, ref. 106) published a series of papers on the movements of ascorbic acid added to defibrinated

[98] F T G Prunty and C C N Vass, *Biochem J* **37**, 623 (1943)
[100] M Pijoan and E Eddy, *J Lab Clin Med* **22**, 1227 (1937)
[101] R Lubschez, *J Clin Invest* **24**, 573 (1945)
[102] T P Cuttle, *Quart J Med* **31**, 575 (1938)
[103]
[104] 353 (1940)
[105]

with the retention of ascorbic acid by leucocytes and platelets floating in the plasma both *in vivo* and, for a considerable time after blood-drawing, *in vitro*, may imply a "dynamic" steady-state retention of ascorbic acid comparable with the accumulation of potassium in the erythrocytes of some species, or a more "static" binding, to protein for example, similar perhaps to the mechanisms by which the B vitamins are in general retained at relatively high concentrations in the leucocytes.

Apart from its effect on hemopoiesis (see p. 437), ascorbic acid has been shown to have a variety of effects on erythrocytes and leucocytes *in vivo* and *in vitro*

Traina[109] in a series of papers has shown that ascorbic acid inhibits the agglutination and affects the hemolysis of human erythrocytes He used very high concentrations in his experiments Merlini has shown that the injection of alloxan plus ascorbic acid into dogs results in grave hemolytic anemia,[110] preceded by methemoglobinemia The normal diabetogenic action of alloxan is not found[110a] (see also p 404). The hemolytic action of dialuric acid has been known for some time (see e g , ref 111), but Merlini's observation points to an investigation of the relation *in vivo* of ascorbone itself to methemoglobin and hemolysis

Massa[112] has shown that at fairly low concentrations ascorbic acid causes an increased oxygen consumption in peritoneal leucocytes of the guinea pig Kovács[113] showed that the phagocytic capacity of leucocytes was increased by glutathione plus ascorbic acid Nungester and Ames[114] found that the phagocytic activity of exudative polymorphs varied directly with their ascorbic acid content and that the quantity and quality of exudates from the peritoneal cavity after an irritating injection ran parallel with the level of vitamin C metabolism of the guinea pig Serum was found to promote phagocytosis and to prevent rupture of cells: the amount needed for a given effect varied inversely with the ascorbic acid in the cells (cf ref 115)

Mills[116] showed that the phagocytic activity for *Micrococcus candidus* of guinea pig granulocytes varied directly with the intake of ascorbic acid His controls were not pair fed.

109 V Traina, *Am J Clin Path* **21**, 141 (1951)
110 D Merlini, *Experientia* **7**, 309 (1951)
110a D Merlini, *Boll soc ital biol sper* **26**, 1248 (1950)
111 F Christensen and H Dam, *Acta Pharmacol Toxicol* **7**, 167 (1951)
112 V Massa, *Bull soc chim biol* **29**, 732 (1947)
113 Z Kovács, *Magyar Biol Kutatóintézet Munkái* **14**, 338 (1942)
114 W J Nungester and A M Ames, *J Infectious Diseases* **83**, 50 (1948)
115 D J Merchant, *Microfilm Abstr* **10**, 6 (1950)
116 C A Mills, *Blood* **4**, 150 (1949)

acid's function in bone formation is further discussed below (see p. 393) It is noteworthy that estrogen tends to inhibit the bone changes of scurvy in the guinea pig.[126]

(5) *Capillaries and Circulation* The effects of vitamin C on the capillaries are closely related to its effects on the other mesenchymal tissues. One of the characteristic signs of scurvy is hemorrhage, and there seems to be no doubt that the maintained integrity of the capillary wall depends on vitamin C Nevertheless, the correlation between capillary strength and plasma ascorbic acid has not been satisfactorily demonstrated in persons outside the frank scorbutic zone, and in some scorbutics capillary strength has been normal (cf p 459)

Since the work of Gothlin,[127] which purported to show that capillary strength was lowered in hypovitaminosis C, many observations have been made on this relation The general opinion is now that there is not a positive or negative correlation between capillary strength and blood ascorbate levels, furthermore, people with scurvy (cf refs 104 and 128) do not always show diminished capillary strength The subject has been reviewed by Munro, Lazarus, and Bell,[129] they conclude that most investigators have failed to demonstrate a relation between capillary strength and chemically assessed vitamin C reserves, and that capillary weakness may persist after cure of scurvy with ascorbic acid

Lee and Lee[130] found that the "spontaneous" bleeding long recognized as a sign of scurvy is always preceded by dilatation of capillaries and venules and that it generally occurs in the non-muscular venules and the venular capillaries The metarterioles and precapillary sphincters appear to be refractory to adrenaline, which appears in the normal animal to control their periodic opening and closing Hemorrhages commonly occur at the site of collagen bundles about the small venules, and Lee and Lee concluded that weakening of surrounding collagen and not of the capillary wall itself was the immediate factor in hemorrhage, and it seems probable that the endothelial intercellular cement is normal in scurvy (see p. 394) The relation of intercellular cement and collagen to vitamin C is discussed below (p 392) Hines and Parker[131] found that in patients with certain vascular diseases, especially with concomitant diabetes, large doses of ascorbic acid may increase capillary strength After abscess formation[132] the growth of capillaries into the lesion in the scorbutic animal is defective.

[illegible]

(1947)

[130] R E Lee and N Z. Lee, *Am J Physiol* 149, 465 (1947).

[131] L E Hines and R J Parker, *Quart Bull Northwestern Univ. Med School* 23, 424 (1949).

[132] E. Meyer and M B. Meyer, *Bull Johns Hopkins Hosp* 74, 98 (1944).

ascorbic acid and the nervous system Watts[120] has shown that ascorbate is oxidized by rat brain homogenates and that this is inhibited by methadone More recently Patterson and Mastin[83] have reported the effect of intravenous ascorbone on the nervous system of the rat It appears that ascorbone has sympathetic (cf ref. 121), parasympathetic, and motor effects, which accompany its entry into the brain and its reduction to ascorbic acid Two hundred milligrams of ascorbone per kilogram of body weight more than doubles the ascorbic acid in the rat brain, raising it from 45 to 116 mg. per 100 g, whereas, rather as in the human erythrocyte and in the eye of the rabbit, ascorbic acid itself has no such effect. Patterson and Mastin suggest that the effect of ascorbone is due to its reduction in the brain and the consequent liberation of protons, which may promote the liberation of "bound" acetylcholine. A lowering of pH certainly inhibits the enzymic and non-enzymic destruction of acetylcholine. A second dose of ascorbone has a very much smaller effect than its predecessor.

(4) *Bone.* Vitamin C is necessary for the formation and repair of the mesenchymal tissues. A very clear statement of the effect of vitamin C on bone formation is given by Follis[122] He finds that in scurvy osteoblasts *fail to lay down dentine on the calcium salts deposited on the cartilaginous* matrix substance and that this calcified substance is not removed, possibly in the way in which the argyrophil characteristics of early collagen are not removed in scurvy (cf p 394) It constitutes the "scorbutic lattice" of Park, and its fracture and disorganization under mechanical stress leads to the classical "Trummerfeldzone." In man the marrow immediately beneath this zone is denuded of hemopoietic cells and occupied by connective tissue cells, forming the so-called "Gerustmark" Follis has made the very important observation that the limb immobilized in plaster is unlike the free limb and shows no fractures, hemorrhage, or "pink-stain-

[illegible]

guinea pig leads to peripheral at the expense of central ossification in bones Murray and Kodicek[124, 124a] find that in guinea pigs with artificial fractures vitamin C appears to control the structure and the density of the new bone laid down Follis[125] has recently made further histochemical studies of the effects of vitamin C deficiency on cartilage and bone The nature of ascorbic

120 D T Watts, *Arch Biochem* 25, 201 (1950).
121 D Merlini, *Acta Vitaminol.* 5, 67 (1951)
122 R H Follis, Jr, The Pathology of Nutritional Disease, Blackwell; Oxford, 1948, p 291
123 G Mouriquand and V. Edel, *Intern Z Vitaminforsch* 20, 392 (1949)
124 *P. D F Murray* and E Kodicek, *J. Anat* 83, 205 (1949).
124a P. D. F. Murray and E. Kodicek, *J. Anat* 83, 285 (1949)
125 R. H. Follis, Jr, *Bull. Johns Hopkins Hosp* [illegible]

represented by Hojer,[144] Fish and Harris,[145] and Ham and Elliott,[146] regards the defect as being in the function of the connective tissue cell

In the course of this controversy, which may be partly a terminological one, Ham and Elliott[146] made the interesting suggestion that the defect in scurvy is failure to form new connective tissue, not an acceleration of a destructive process, and, in spite of the value of modern ideas of dynamic steady-state processes, the work of Neuberger, Perrone, and Slack[147, 147a] on the rat supports this conclusion in that some collagen, once formed, appears to be almost closed to further metabolic exchange Hunt[148] obtained some evidence that collagen in newly formed scars reverts during scurvy—a result not supported by recent work[148a] on collagen and ascorbic acid—and named the substance to which this scurvy-sensitive new collagen reverts "precollagen" According to Wolbach's ideas precollagen would be provided normally by the scorbutic animal and the defect would lie in its non-conversion, outside the cell, to collagen Wolbach and Bessey[143] argued that the "pink-staining" amorphous extracellular substance in scorbutic tissues is the precursor of collagen, but Follis,[122] from his work on bones protected from mechanical stress (see p 392), showed that its presence at least in bone, depends on stress He regards Wolbach's gelation theory as unproved

There is some evidence that the precursor of normal collagen has a polysaccharide constituent Thus, from an examination of the effect of ascorbic acid on wound-healing in guinea pigs, Penney and Balfour[149] confirmed by metachromatic staining with toluidine blue the production of acid mucopolysaccharides in the early stages of wound-healing in normal guinea pigs In C-depleted animals, cells had migrated into the clot, and though there was fibroblastic proliferation the fibroblasts were abnormal They were scarcely ever associated to form syncytia, and there was very little intercellular material Capillary proliferation was limited. Within 12 hr of injection of ascorbic acid normal fibroblasts were present, there was much extracellular material, and fine reticulin fibers appeared Hyaluronidase was used to show that the extracellular material consisted partly of acid mucopolysaccharides of the hyaluronic acid or chondroitin sulfate type More recently Bradfield and Kodicek[150] have confirmed earlier work that

[144] J A Hojer, *Acta Paediat Suppl* **3**, 1 (1924)
[145] E W Fish and L J Harris, *Trans Roy Soc (London)* **B233**, 489 (1934)
[146] A W Ham and H C Elliott, *Am J Path* **14**, 323 (1938)
[147] A Neuberger, J C Perrone, and H G B Slack, *Biochem J* **49**, 199 (1951)
[147a] A Neuberger and H G B Slack, *Biochem J* **53**, 47 (1953)
[148] A H Hunt *Brit J Surg* **28**, 436 (1941)
[148a] W van B Robertson, *J Biol Chem* **196**, 403, **197**, 495 (1952)
[149] J R Penney and B M Balfour, *J Path Bact* **61**, 171 (1949)
[150] J R G Bradfield and E Kodicek, *Biochem J* **49**, xvii (1951)

Capillary permeability, unlike strength, is probably a function of the intercellular cement (cf ref 133), which is probably independent of ascorbic acid In the whole animal ascorbic acid appears to limit transport from blood through the capillary wall, protecting guinea pigs against hemorrhagic shock,[134] increasing the rise in oncotic pressure, but not in serum protein, of rabbits recovering from plasmapheresis,[135] and reducing transudation of fluid into serous cavities in dogs injected with trypan blue [136] Elster and Schack[137] concluded from a study of plasma concentrations of Evans blue (T1824) in the normal and scorbutic guinea pig that capillary permeability is not altered, but they found that extravasation of the dye into the periarticular soft tissues is frequent in scurvy They regard the relation of ascorbic acid to the endothelial cement as unknown

Sheppard and McHenry[138] have claimed that water retention is decreased in the C-deficient guinea pig, and Keller and Kunzel[139] have stated that ascorbic acid modifies the surface activity of lecithin and lecithin-cholesterol mixtures Among miscellaneous observations on the circulation are that of Regidor,[140] who states that intravenous ascorbic acid is vasodilator, that of Mundo Fuertes and Valle Cobo,[141] who report that ascorbic acid given to the dog increases the absolute erythrocyte count in venous blood from the spleen, and that of Allardyce, Fitch, and Semple,[142] who state that the feeding of histidine with ascorbic acid to rats prevents the rise in blood pressure normally following an injection of desoxycorticosterone-acetate The degeneration of the myocardium in scurvy is an example of general muscular degeneration (p 443)

(6) *Collagen, Intercellular Cement, etc* It is generally agreed that connective tissue is defective in the scorbutic animal [122, 143] There is disagreement about the exact nature of the defect, the Wolbach school having taken the view that the fibroblast or similar connective tissue cells can secrete normally, but that ascorbic acid is ultimately necessary for the setting or fibrillation of collagen and similar substances Another group,

[133] R Chambers and B W Zweifach, *Physiol Revs* **27**, 436 (1947)
[134] C D de Pasqualini, *Am J Physiol* **147**, 598 (1946)
[135] S Mori, *Tôhoku J Exptl Med* **49**, 247 (1948)
[136] J Kiersz, *Arch intern pharmacodynamie* **81**, 479 (1950)
[137] S K Elster and J A Schack, *Am J Physiol* **161**, 283 (1950)
[138] M Sheppard and E W McHenry, *Biochem J* **33**, 655 (1939)
[139] C J Keller and O Kunzel, *Z ges exptl Med* **103**, 704 (1938)
[140] P de la Peña Regidor, *Rev clin esp* **40**, 176 (1951)
[141] A Mundo Fuertes and M del Valle Cobo, *Trabajos inst nacl cienc méd (Madrid)* **7**, 499 (1945–1946)
[142] J Allardyce, F. Fitch, and R Semple, *Trans Roy Soc Can* III, **42**, 25 (1948)
[143] S B Wolbach and O A Bessey, *Physiol Revs* **22**, 233 (1942)

chromatic-staining material containing polysaccharides, and the fiber formation appears defective, but it is not certain whether the defect is in extracellular gelation or in the production of a precursor or factor by the connective tissue cell It may well be that ascorbate or its oxidation products are concerned in the removal of polysaccharides in the later stage of collagen formation, or are catalytic in setting up cross-linkages, such as —S—S— bonds, between protein chains in collagen They may also be concerned in the oxidation of organic sulfur to sulfate in the production of chondroitin sulfate Barrenscheen and Valyi-Nagy[157] have implicated ascorbate in the oxidation of the sulfur of methionine (p 439)

Species differences make it difficult to integrate the effects of cortisone and corticotropin, which inhibit wound-healing,[158] with the effects of ascorbic acid If cortisone causes a high ascorbone-ascorbate ratio (p 417) and if it is accepted that a high ratio is found in scurvy (p 382), the similar effects on wound-healing of scurvy and cortisone may perhaps be explained Further discussion of this difficult set of factors, which should probably include the thyroid and the mast cells[159, 159a] will be found on p 408 It is thought by Pirani *et al*[159b] that the action of ascorbate in wound-healing is direct and not mediated by the adrenal cortex.

Roche, Nataf, and Marois[160] have shown that the guinea pig with scurvy responds to relaxin

(7) *Mouth* Bleeding and degeneration of the gums were long regarded as classical signs of scurvy, but recent work on pure vitamin C deficiency in man[104, 125] has shown that gum changes are frequently but not always part of the acute syndrome (see p 460 for work on monkeys) Wolbach and Bessey[143] and Follis[125] regard changes in gums, bone, and teeth as secondary to the main defect of scurvy—failure to form connective tissues. Calcification is not deficient in scurvy, though structural changes in the rapidly growing incisors of guinea pigs are seen early in scurvy King[161] has reviewed the relation of ascorbic acid to the mouth He does not accept Kruse's[162] contention that "chronic" slight C deficiency leads to virtually irreversible gum changes Bronstein[163] has found that a reduced oxygen pressure promotes paradontosis and scurvy in the guinea pig and possibly

[157] H K Barrenscheen and T von Valyi-Nagy, *Z physiol Chem* 283, 91 (1948).
[158] J W Blunt, Jr, C M Plotz, F Lattes, E L Howes, K Meyer, and C Ragan, *Proc Soc Exptl Biol Med* 73, 678 (1950)
[159] G Asboe Hansen, *Acta Dermato-Venereol* 30, 338 (1950)
[159a] G Asboe Hansen, *J Invest Derm* 15, 25 (1950)
[159b] C L Pirani, R C Stepto, and C F Consolazio, *Federation Proc* 11, 423 (1952)
[160] J Roche, B Nataf, and M Marois, *Ann endocrinol Paris* 12, 212 (1951)
[161] J D King, *Nutrition Abstracts & Revs* 17, 569 (1948)
[162] H D Kruse, *Milbank Mem Fund Quart* 20, 290 (1942)
[163] V E Bronstein, *Am Rev Soviet Med* 1, 314 (1943-1944)

in wounds in scorbutic guinea pigs either connective tissue fails to regenerate or else abnormally thick precollagen argyrophil strands accumulate Periodate-Schiff staining, which is very probably specific for polysaccharides, is virtually absent from mature collagen in wounds in normal animals In scurvy, wounds show thick, chaotically ramifying precollagen strands, sheathed in material strongly positive to periodate-Schiff stain. This material, probably polysaccharide, was largely not sulfated and was not removed by amylase or hyaluronidase. They think it highly probable that the argyrophilia of the precollagen of scurvy and of the young reticular fibers in normal animals is due to polysaccharides

Elster[151] has concluded from the collagen contents of scorbutic and normal guinea pigs of the same weight that maintenance of collagen does not require ascorbic acid. This conclusion is confirmed by the similar experiments of Robertson[148a, 152] and supported by Burns, Burch, and King,[153] who showed that 1-C^{14}-L-ascorbic acid is not found in the chondroitin sulfate or collagen fractions from cartilage or skin, all the C^{14} in nasal septum or skin was readily extracted with water or 8% acetic acid, and all the C^{14} in the nasal septum was present in ascorbic acid The highest C^{14} activity per milligram of carbon was found in the incisor and molar teeth, Robertson[152] had found a small reduction of collagen only in the teeth, kidney, and costochondral junctions of scorbutic guinea pigs. The incisors of the guinea pig are continually growing, and this may account for their high C^{14} activity in normal guinea pigs and their low collagen in scorbutic guinea pigs

Ascorbic acid promotes the formation of fibers in cultures of fibroblasts from chick embryos,[154] but it did not promote the production of collagen in guinea pig fibroblast cultures[155] Finally, Chambers and Cameron[156] found that ascorbate was not necessary for the cohesiveness of sheets of epithelial cells and concluded that ascorbic acid was not necessary for formation of intercellular cement in such tissue cultures How comparable conditions in tissue culture are with those in the body is doubtful; striking differences would appear to be the higher oxygen pressure and the absence of mechanical stress in the former.

In summary, it appears that connective tissue can be maintained, but not replaced, in the scorbutic guinea pig, and that the 1-carbon of ascorbic acid is not incorporated into this tissue except as easily extracted ascorbate. In the wounded scorbutic guinea pig there is excessive production of meta-

151 [illegible] (1950)
152 [illegible]
153 [illegible] (1951).
154 [illegible] (1936–1937)
155 [illegible]
156 [illegible]

The 5 g of ascorbic acid in the "saturated" body is thus a steady-state level which is a function primarily of intake and secondarily of excretion and metabolism The question whether a high level is "desirable" is further discussed on p 461

It is clear that the "saturation" test, i e , the measurement of the ingested ascorbic acid needed to cause a large excretion, measures to about 0 5 g the difference between the initial body content and the content at which excretion is high This can give only a very rough estimate of the position of the subject with respect to the first gram of ascorbic acid—the one that includes the small amount that prevents scurvy—and when the test is completed no further investigation of this original position is possible There seems to be very little, if any, more information to be derived from a saturation test than from an estimation of the plasma level (cf. p 380) the estimation of ascorbate in the leucocytes and platelets is more valuable than either The close correlation between the results of saturation tests and plasma estimations has been shown by Prunty and Vass[98] and Ericsson [173]

The physiology of the excretion of vitamin C has been reviewed recently by Smith [174] It is a threshold substance, and the mechanism of its reabsorption in the tubule is not understood A speculative suggestion that it may depend on oxidation and reduction in different parts of the tubule or tubular cell is given below (pp 407–408)

The renal threshold in man was first demonstrated by Ralli, Friedman, and Rubin,[175] who showed that in three subjects the average tubular reabsorption maximum was 1 77 mg per 100 ml of tubular filtrate per minute or 2 16 mg per minute This rate was unaffected by variation in the flow of urine from 1 5 to 15 ml per minute or by raising the blood glucose above 300 mg per 100 ml and thus exceeding the tubular reabsorption maximum for glucose Sherry *et al* [176] showed that the reabsorption figure in three dogs averaged 0 52 mg per 100 ml filtrate per minute The dog is independent of dietary ascorbic acid

Ascorbic acid is not as clear-cut a threshold substance as glucose Friedman, Sherry, and Ralli[177] showed that at plasma levels of 0 03 to 1 4 mg per 100 ml the clearance stabilizes at 1 to 3 ml per minute and that reabsorption is never complete This implies, as urine flow is usually about 1 ml per minute, that the concentration of ascorbic acid in urine is seldom less than that in plasma

[173] E Ericsson, *Acta Paediat* 28, 140 (1939)

[174] H W Smith, The Kidney, Oxford University Press, New York, 1951

[175] E P Ralli, G J Friedman, and S H Rubin, *J Clin Invest* 17, 765 (1938)

[176] S G Sherry, G J Friedman, K Paley, J Berkman, and E P Ralli, *Am J Physiol* 130, 276 (1940)

[177] G J Friedman, S Sherry, and E P Ralli, *J Clin Invest* 19, 685 (1940)

also in man The teeth of the guinea pig show the earliest tissue changes in scurvy [71]

(8) *Skin* In 1937 Rotter[165] proposed that 2,6-dichlorophenol indophenol could be injected intradermally in man to measure C nutriture. The test has not met with general approval Very little is known of concentrations of ascorbic acid in skin. Its role in the formation of skin depends on its relation to the formation of connective tissue in general Work on skin wounds has already been discussed

(9) *Liver* A few miscellaneous observations have been made on ascorbic acid in the liver Little appears to be known of the concentrations of ascorbate in the portal and hepatic venous blood streams after ingestion, and (see p 380) liver contains not more than about thirty times as much ascorbate as blood[71] in the guinea pig

Adrenalectomy initially decreases and finally raises ascorbate in the liver of mice[166] Lindan and Work[167] find that liver necrosis resulting from a diet deficient in S-containing amino acids is accompanied by a fall in ascorbate The liver of animals on a stock diet contained 48 mg ascorbic acid per 100 g, on a necrogenic diet 26 mg. ascorbic acid per 100 g, and after necrosis no ascorbic acid The decrease in ascorbate was accompanied by a decrease in the amount of reduced glutathione, the oxidized form remaining approximately constant

b. Secretions. (1) *Urine* Early work on urinary ascorbic acid was largely concerned with the use of the excretion of ascorbic acid, usually after oral dosage, as an indicator of nutriture in respect of the vitamin [169] Wright, Lilianfeld, and MacLenathen[170] used excretion after an intravenous test dose for this purpose A large variety of these so-called "saturation" tests have been used and described, the main variations being in the size of the dose, in the times of collection of urine, and in the use of single or repeated doses It is difficult to decide whether or not the concept of "saturation" of the body with a nutrient is trivial (cf pp 460–464) It appears, from post-mortem analyses and from the amounts ingested by a scorbutic person before copious excretion ensues, that the adult human body can by dietary methods be caused to contain about 5 g of vitamin C Scurvy may be dispelled by the administration to the scorbutic subject of less than 1 g of ascorbic acid There is evidence[171] that the metabolism of ascorbic acid in the body varies directly with the average intake, and it is certain that the excretion varies in the same way

[165] H Rotter, *Nature* 139, 717 (1937)
[166] W. Muller, *Arch path Anat* (*Virchow's*) 320, 174 (1951)
[167] O Lindan and E Work, *Biochem J* 48, xxxi (1951)
[169] M A Abbasy, L J Harris S N Ray, and J R Marrack, *Lancet* 229, 1399 (1935)
[170] I S Wright, A Lilianfeld, and E MacLenathen, *Arch Internal Med* 60, 264 (1937)
[171] M van Eekelen, *Biochem J* 30, 2291 (1936).

The reabsorptive mechanism for ascorbate is probably partly in the proximal tubule, in which glucose is largely absorbed.[174] This view concurs with the histological demonstration by Giroud and Leblond[184] that ascorbic acid is found only in the proximal tubules and the descending limb of Henle's loop after its intravenous administration to guinea pigs. It must be remembered that their method uses silver nitrate and presumably does not detect ascorbone.

Hawthorne and Storvick[185] have shown that sodium bicarbonate lowers blood and urinary ascorbate in man and that ammonium chloride lowers blood but raises urinary ascorbate. Boutwell *et al.*[186] reported that in six subjects getting 91 mg. ascorbic acid per day and exposed to pressures simulating an altitude of 18,000 ft. the excretion of ascorbate was decreased from an average of 49 to 28 mg. per day. There was no accompanying change in tolerance to phenylalanine. Piantoni[188] showed that phlorizin partly impairs reabsorption of ascorbate in the rabbit. With Orlas[189] he claimed that progesterone and desoxycorticosterone reduced excretion of ascorbate, apparently via the filtration rate, in the rabbit, and Selkurt, Talbot, and Houck[190] showed that large amounts of estradiol benzoate decreased tubular reabsorption in female dogs, which showed eventually increased capillary fragility, petechial hemorrhages, increased clotting times, and, in some, fatal anemia. The reduction in absorption is due not to an absolute reduction in Tm at very high plasma (i.e., very high load per Tm) values but to a reduction in absorption at normal plasma values (cf. ref. 174). Demole[191] has shown that the adult male Swiss mouse excretes urine containing 50 to 100 mg. per 100 ml. and that the female urine contains half this amount. Diet and the administration of sex hormones did not affect these values.

(2) *Milk.* The concentrations of ascorbic acid in the milk of scurvy-liable and scurvy-resistant species have been used in the controversy on vitamin C requirements (see ref. 2 and p. 460). Table 8 summarizes some of the results now available.

The scurvy-liable animals show clear evidence that ascorbate is in effect secreted into the milk from the blood. In the scurvy-resistant animals in which the milk level is higher than the serum or plasma level there may be secretion and/or synthesis in the mammary gland. Braude, Kon, and

[184] A. Giroud and C. P. Leblond, *Anat. Record* 68, 113 (1937).
[185] B. E. Hawthorne and C. A. Storvick, *Proc. Soc. Exptl. Biol. Med.* 67, 447 (1948).
[186] J. H. Boutwell, J. H. Cilley, L. R. Krasno, A. C. Ivy, and C. J. Farmer, *J. Applied Physiol.* 2, 388 (1950).
[188] C. Piantoni, *Rev. soc. argentina biol.* 16, 175 (1940).
[189] C. Piantoni and O. Orlas, *Rev. soc. argentina biol.* 17, 153 (1941).
[190] E. E. Selkurt, L. J. Talbot, and C. R. Houck, *Am. J. Physiol.* 140, 260 (1943).
[191] V. Demole, *Helv. Physiol. Pharmacol. Acta* 5, C40 (1947).

Ahlborg,[178] who has made the most elaborate study of the excretion of ascorbic acid in man, has plotted total urinary excretion against plasma concentration and so measured the filtration fraction. The average for 13 subjects was 131 ml per 1.73 sq.m. body surface per minute, as compared with a mean of 124 for inulin clearance in the same subjects, and 123 as given by Smith[174] as a mean for 157 subjects. Ahlborg's 13 subjects showed no correlation between results from inulin and from ascorbic acid The use of oral vitamin C for measuring filtration rate has advantages as it involves no intravenous administration and the analytical techniques are relatively easy Smith warns against the use of "approximation" techniques of this type, partly because the glomerular filtration rate may alter between the measurements at the two super-threshold plasma levels needed. The interconversion of ascorbate and ascorbone may take place in the kidney and complicate the comparison of concentrations in urine and plasma

Ahlborg[178] made the interesting observation that when the plasma level of ascorbate was maximal after injection the excretion was not maximal but lagged by some 20 minutes This observation, which he attributed to storage of ascorbic acid in the kidney, *means that the tubular reabsorption* maximum for ascorbic acid is high during the lag phase There is a formula for compensating saturation tests for "renal retention" of ascorbic acid [179]

The relations between the excretion of ascorbic acid and that of other solutes have been examined As mentioned above, Ralli *et al.*[175] and Sherry *et al* [176] claimed that saturation of the $Tm_{glucose}$ in man and dog respectively does not affect $Tm_{ascorbate}$, but Selkurt[180] found that maintained saturation partly blocked the reabsorption of ascorbate, with partial recovery later He found a similar blockage and recovery after saturation with *p*-aminohippuric acid, an effect adding to that from glucose, though these latter two solutes only slightly interfere with each other's reabsorption Selkurt and Houck[181] found that infusion of sodium and potassium chlorides reduced $Tm_{ascorbate}$ and Stamler[182] has shown that ascorbic acid levels do not affect the excretion of sodium or potassium in the dog It thus appears that the absorption of ascorbic acid is affected only indirectly by the absorptions of other substances These absorptions may share common sources of energy *Mannitol diuresis does not reduce* $Tm_{ascorbate}$[181] and ascorbic acid itself may be a diuretic agent (cf. ref 183 and 183a)

[178] N G Ahlborg, *Acta Physiol Scand* **12**, *Suppl* 36 (1946).
[179] J B Ludden and I Wright, *Arch Internal Med* **65**, 151 (1940)
[180] E E Selkurt, *Am J Physiol* **142**, 182 (1944)
[181] E. E Selkurt and C R Houck, *Am J. Physiol* **141**, 423 (1944)
[182] [illegible] 155, 100 (1951)
[183] [illegible]
[183a] [illegible] m, and A - W.El-Sheeby, *Intern. Z Vitaminforsch* 24, 40 (1952)

(3) *Semen* Concentrations of ascorbate in semen have been found by Nešpor[206] to be 3 mg per 100 ml for man and 5 mg per 100 ml for the guinea pig Mann[207] gives figures of 12 mg per 100 ml for man, 3 to 8 for the bull, and 8 for the guinea pig Berg, Huggins, and Hodges[208] have shown that in man and in the guinea pig ascorbate in the semen is much higher than in the plasma In the dog, which unlike man has no seminal vesicle, the semen level is similar to that of plasma Table 9 gives their figures for concentrations in various parts of the ejaculate of man.

TABLE 9

ASCORBIC ACID IN SEMINAL CONSTITUENTS OF MAN

	No of samples	Ascorbic acid, mg /100 ml
Ejaculate	9	12 8
Seminal vesical fluid	9	4 7
Seminal vesical + prostatic fluid	11	2 4
Prostatic fluid	19	0 54
Spermatocele fluid	10	0 97

(4) *Sweat, Saliva, and Gastric Secretion* There is very little ascorbic acid in sweat, though copious sweating may result in significant loss[209, 210]

Glavind *et al*[211] confirmed earlier work on ascorbic acid in saliva, finding about 0 2 mg per 100 ml Hess and Smith[212] found 0 21 mg per 100 ml, in caries-free subjects and 0 17 mg per 100 ml in those with caries Freeman and Hafkesbring[213] found for 110 normal subjects the value of 0 07 mg per 100 ml They found 0 98 mg per 100 ml, of whole blood, 1 59 mg per 100 ml of urine, and an average daily excretion of 14,15 mg in the same subjects The mean concentration for gastric secretion was 1 05 mg per 100 ml during fasting and 0 91 mg per 100 ml after stimulation Though the means for blood and gastric secretion are close, there was no correlation between individual pairs of values this was taken to indicate that the secretion of ascorbic acid is a physiological property of the cells of the gastric mucosa It must be remembered that whereas in blood ascorbic acid is largely ionized, in gastric secretion it is largely un-ionized

[206] E Nešpor, *Klin Wochschr* **18**, 135 (1939)

[207] T Mann, *Advances in Enzymol* **9**, 329 (1949)

[208] O C Berg, C Huggins, and C V Hodges, *Am J Physiol* **133**, 82 (1941)

[209] I S Wright and E MacLenathen, *J Lab Clin Med* **24**, 804 (1939)

[210] J B Shields, B C Johnson, T S Hamilton, and H H Mitchell, *J Biol Chem* **161**, 351 (1945)

[211] J Glavind H Granados [illegible] H Dam, *Intern* [illegible]

[212] W C [illegible]

[213] J T [illegible]

Porter[204] have shown that the pregnant sow's plasma ascorbate is about 0 7 mg per 100 ml and does not change after farrowing; piglets can survive on goat's milk without supplements of ascorbic acid, and they appear to be independent of dietary supplies, in spite of the high concentrations in the milk and colostrum of sows

TABLE 8

CONCENTRATIONS OF ASCORBIC ACID IN MILK AND COLOSTRUM (MG /100 ML)

Species	Milk	Colostrum	Reference
Woman	ca. 7		192
	ca 4	5	193
Sow, dry lot	10 4	18 8	194
Sow, pasture	12 2	24 6	194
Percheron mare	15 0	5 6	195
Mare	12 1		196
	15 7		197
Goat	1 8		198
	0 9		199
Guinea pig	12–80		200
Cow	0 98 "free", 0 6 "combined"		201
	0 7–6 0	12 5	2
Sheep	0.80	5 9	202
Buffalo	1		2

The concentration in human milk reaches a plateau of about 7 mg per 100 ml at a level of intake giving urinary saturation [192] The output of vitamin C in the milk may thus reach 70 mg per day, a high intake relative to the infant's body weight of perhaps 8 kg at the time of highest milk consumption

[192] I Selleg and C G King, *J Nutrition* **11**, 599 (1936)

[193] S K Kon and E H Mawson, Human Milk, Medical Research Council, Special Report Series No 269, H M S O , London, 1950, 188 pp

[194] J P Bowland, R H Grummer, P H Phillips, and G Bohstedt, *J Animal Sci* **8**, 98 (1949) [*Nutrition Abstracts & Revs* **19**, 55 (1949)]

[195] A D Holmes, A F Spelman, and R T Wetherbee, *J Nutrition* **37**, 385 (1949)

[196] A D Holmes, *Arch Biochem* **27**, 125 (1950)

[197] A Cimmino, *Ann igiene* **50**, 471 (1940), cited in ref 2, p 144

[198] A D Holmes, H G. Lindquist, and E J Finnegan, *J. Am Dietet Assoc* **26**, 179 (1950)

[199] R. K Chakraborty, *Indian J Med Res* **23**, 347 (1935)

[200] R Rasmussen, R Bogart, and L A. Maynard, *Proc Soc Exptl Biol Med* **39**, 502 (1938)

[201] [illegible]

[202] [illegible] *J Nutri-* [illegible]

[illegible] 1950)

[204] [illegible]

(3) *Semen* Concentrations of ascorbate in semen have been found by Nešpor[206] to be 3 mg per 100 ml for man and 5 mg per 100 ml for the guinea pig Mann[207] gives figures of 12 mg per 100 ml for man, 3 to 8 for the bull, and 8 for the guinea pig Berg, Huggins, and Hodges[208] have shown that in man and in the guinea pig ascorbate in the semen is much higher than in the plasma In the dog, which unlike man has no seminal vesicle, the semen level is similar to that of plasma Table 9 gives their figures for concentrations in various parts of the ejaculate of man

TABLE 9
ASCORBIC ACID IN SEMINAL CONSTITUENTS OF MAN

	No. of samples	Ascorbic acid, mg /100 ml
Ejaculate	9	12 8
Seminal vesical fluid	9	4 7
Seminal vesical + prostatic fluid	11	2 4
Prostatic fluid	19	0 54
Spermatocele fluid	10	0 97

(4) *Sweat, Saliva, and Gastric Secretion* There is very little ascorbic acid in sweat, though copious sweating may result in significant loss [209, 210] Glavind et al [211] confirmed earlier work on ascorbic acid in saliva, finding about 0 2 mg per 100 ml Hess and Smith[212] found 0 21 mg per 100 ml in caries-free subjects and 0 17 mg per 100 ml in those with caries Freeman and Hakfesbring[213] found for 110 normal subjects the value of 0 07 mg per 100 ml They found 0 98 mg per 100 ml of whole blood, 1 59 mg per 100 ml of urine, and an average daily excretion of 14 15 mg in the same subjects The mean concentration for gastric secretion was 1 05 mg per 100 ml during fasting and 0 91 mg per 100 ml after stimulation Though the means for blood and gastric secretion are close, there was no correlation between individual pairs of values this was taken to indicate that the secretion of ascorbic acid is a physiological property of the cells of the gastric mucosa It must be remembered that whereas in blood ascorbic acid is largely ionized, in gastric secretion it is largely un-ionized

206 L Nešpor, *Klin Wochschr* 18, 135 (1939)
207 T Mann, *Advances in Enzymol* 9, 329 (1949)
208 O C Berg, C Huggins, and C V Hodges, *Am J Physiol* 133, 82 (1941)
209 I S Wright and E MacLenathen, *J Lab Clin Med* 24, 804 (1939)
210 J B Shields, B C Johnson, T S Hamilton, and H H Mitchell, *J Biol Chem* 161, 351 (1945)
211 J Glavind H Granados, L A Hansen, K Schilling, I Kruse, and H Dam, *Intern Z Vitaminforsch* 20, 234 (1948)
212 W C Hess and B T Smith, *J Dental Research* 28, 507 (1949)
213 J T Freeman and R Hafkesbring, *Gastroenterology* 18, 224 (1951)

c. Absorption. Human fecal losses are normally less than 5 mg. per day,[214] but catharsis and diarrhea have been shown to hinder the absorption of ascorbic acid in infants[215] Todhunter and Fatzer[216] have shown that crystalline ascorbic acid and ascorbic acid in raspberries are equally utilized by women Penney and Zilva[70] have shown that the absorption of ascorbic acid is much more complete in man than in the guinea pig

2. Hormonal Aspects

a. Carbohydrate Metabolism. In 1936 Sigal and King[217] showed that scorbutic guinea pigs had a reduced glucose tolerance Depletion for about 10 days was needed to show the effects, and these could be cleared up, after depletion for 20 days, by 10 mg of ascorbic acid per day.

In 1944 Banerjee[218] showed that the islets of Langerhans increase in number and size in scorbutic animals, and with Ghosh in 1946[219] that the scorbutic decrease in glucose tolerance was probably not due to increased secretion of adrenaline (cf pp 418 and 449.) They showed in 1947[219] that the decreased tolerance was accompanied by a lowered deposition of liver glycogen and by a lowered pancreatic content of insulin, and in 1949[220] that it was not due to hypo- or hyperthyroidism (cf p 421) Banerjee and Deb[221] have shown that adrenal cholesterol is reduced in scorbutic guinea pigs, and they argue that adrenal cortical function is lowered They[222] have also shown that excretion of 17-ketosteroids is reduced in scurvy. Their suggestion that adrenal cortical function is reduced and that this reduction contributes to the lowered glucose tolerance appears self-contradictory The bulk of the evidence (see p 410) supports an increased or normal function in scurvy

Murray[223] has investigated some of the enzymes of carbohydrate metabolism in the scorbutic guinea pig The liver's ability to phosphorylate was reduced in scurvy, and its oxygen consumption increased Her earlier work with Morgan[224] showed that under air at 349 mm Hg for 24 hr, which caused anoxic anoxia, deficient guinea pigs, unlike normals, maintained or increased their carbohydrate stores This suggests a relation between the

214 C J Farmer, A F Abt, and H Chinn, *Quart. Bull Northwestern Univ Med School* 14, 114 (1940)
215 A. F Abt, C J Farmer, and Y J Topper, *Proc Soc Exptl. Biol. Med* 43, 24 (1940)
216 E. N Todhunter and A S Fatzer, *J Nutrition* 19, 121 (1940)
217 A Sigal and C G King, *J Biol Chem* 116, 489 (1936)
218 S Banerjee, *Nature* 153, 344 (1944)
219 S. Banerjee and N. C Ghosh, *J Biol Chem* 166, 25 (1946), 168, 207 (1947)
220 S Banerjee and N C Ghosh, *J Biol Chem* 180, 189 (1949)
221 S Banerjee and C Deb, *J Biol Chem* 190, 177 (1951).
222 S Banerjee and C Deb, *J Biol Chem* 194, 575 (1952)
223 H. C Murray, *Proc Soc. Exptl Biol Med* 75, 598 (1950).
224 H. C. Murray and A F. Morgan, *J Biol Chem* 163, 401 (1946)

lowered carbohydrate store and the increased oxygen consumption of the liver of the deficient guinea pig kept in ordinary air

Kiverin and Kiverina[225] showed that in guinea pigs and rabbits a higher and more prolonged rise in blood sugar was caused by injection of adrenaline into animals saturated with vitamin C than in others In six human subjects saturation with vitamin C caused no significant difference in adrenaline hyperglycemia (see also ref 226 and p 417)

Daoud and El Ayyadi[227] obtained results on the rat which indicated that insulin affects the distribution or synthesis of ascorbic acid and that ascorbic acid in some way potentiates adrenaline (see also p 418) Sherry and Ralli[228] suggest that in man, dog, and rat insulin causes movement of ascorbic acid from plasma to the tissues Ascorbic acid thus appears to resemble glucose and galactose [229]

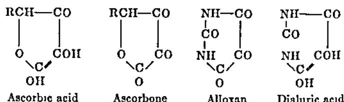
Nath and Chakrabarti[230] have shown that rabbits after repeated injections of acetoacetate for 60 days showed a reduction of plasma ascorbic acid from 2 5 to 0 83 mg per 100 ml The fall in the ascorbic acid in the plasma was accompanied by a rise in blood lactate Injected acetoacetate aggravates the defect in carbohydrate metabolism of scorbutic guinea pigs.[230a]

Vogt[231] has shown that in rats deprived of the adrenal medulla the adrenal ascorbic acid falls after insulin hypoglycemia It seems that this effect is a corticotropin effect (see p 411), possibly overcoming the direct effect of insulin that might be expected from the results of Sherry and Ralli [228] Vogt's experiment was designed to show that the adrenal medulla is not essential for hypoglycemic stress to affect the metabolism of the adrenal cortex The effect of insulin on adrenal ascorbic acid during artificial maintenance of the blood sugar is not apparently known

The "near-diabetic" state of the scorbutic guinea pig may be related to the extremely important observation of Patterson[232, 233] that the injection of ascorbone (prepared by oxidation of ascorbic acid with quinone) leads to toxic effects (cf ref 83 and p 390) and under certain conditions to a permanent diabetic state closely resembling alloxan diabetes The toxicity of certain carbohydrate derivatives and of certain pyrimidines has been known for some time, but their mode of action is not fully known

[225] M D Kiverin and A A Kiverina, *Fiziol Zhur S S S R* **36**, 621 (1950)
[226] M D Kiverin *Biokhimiya* **16**, 222 (1951)
[227] K M Daoud and M A S El Ayyadi, *Biochem J* **30**, 1280 (1936)
[228] S Sherry and E P Ralli, *J Clin Invest* **27**, 217 (1948)
[229] R Levine M S Goldstein B Huddlestun, and S P Klein, *Am J Physiol* **163**, 70 (1950)
[230] C Nath and C H Chakrabarti, *Proc Soc Exptl Biol Med* **78**, 2?? (1951)
[230a] M C Nath, A K Sahu and R P Chitale, *Biochem J* **53**, 681 (1953)
[231] M Vogt *J Physiol* (*London*) **114**, 222 (1951)
[232] J W Patterson, *Endocrinology* **45**, 344 (1949)
[233] J W Patterson, *J Biol Chem* **183**, 81 (1950)

In general, toxicity is very specifically related to chemical structure, and Patterson was led to investigate ascorbone by its similarity to alloxan:



Ascorbic acid Ascorbone Alloxan Dialuric acid

Hewitt[39a] gives for the alloxan-dialuric acid system $E_b = 0\,06 + 0\,03 \frac{[\text{alloxan}]}{[\text{dialuric acid}]}$ V at 30° and pH 7 0 and points out that the two reactants combine to give alloxantin, analogous with a meriquinone

Ball[39] found E_0 at 30° and pH 7 24 for ascorbic acid and ascorbone to be +0 051V The closeness of this value to that of the alloxan system is striking, and the question of a meriquinone of ascorbic acid arises (see p 422)

The mechanism of alloxan diabetes has been discussed by Lazarow[237] He regards glutathione as a key metabolite which may be so seriously reduced in the islet cells by alloxan as to cause such interference with —SH enzymes and —SH compounds that irreversible damage ensues —SH compounds administered before or with alloxan protect rats against diabetes[239]

Lazarow stresses the high oxidation-reduction potential of the beta cells as demonstrated by their slow reduction of Janus green This is consistent with their synthesis of insulin, a sulfur-rich compound with all its sulfur in the disulfide form.[241] The protective action of glutathione may be due to *in vivo* reduction of alloxan in the blood or to the accumulation of a "protective" amount of glutathione in the beta cells before alloxan enters them Lazarow's[237] hypothesis appears to be, in short, that —SH compounds can reduce alloxan before it has time actually to oxidize or combine with the —SH compounds in the beta cells: the effect of alloxan cannot be reversed by glutathione given subsequently

Lazarow[239] and Levey and Suter[242] have found, however, that ascorbic acid, like glutathione a physiological reducing agent, enhances the diabetogenic effects of alloxan, although Merlini[110a] claims that ascorbic acid can prevent alloxan diabetes Lazarow[239] invokes Prunty and Vass's observation[242a] that plasma ascorbic acid and erythrocyte glutathione are

[237] A Lazarow, *Physiol Revs* **29**, 48 (1949)
[239] A Lazarow, *Proc Soc Exptl Biol Med* **61**, 441 (1946)
[241] V du Vigneaud, *J Biol Chem* **75**, 393 (1927)
[242] S Levey and B Suter, *Proc Soc Exptl Biol Med* **63**, 341 (1946)
[242a] F T. G Prunty and C C N Vass, *Biochem J* **37**, 506 (1943)

inversely related in man (but in comparatively long-term experiments) to explain this, by arguing that the ascorbic acid reduces the tissue glutathione, in particular that of the beta cells, but in his short-term experiments it seems unlikely that this factor would be operative Furthermore, Banerjee, Deb, and Belavady[78] have shown that glutathione is diminished in blood, adrenals, spleen, and (particularly) pancreas in scorbutic guinea pigs.

In elaborating his hypothesis, Lazarow[277] apparently has not taken account of the important observation of *Jimenez Diaz, Grande Covián,* and De Oya[340] that *dogs* were completely protected against the diabetogenic and nephrotoxic effects of injected alloxan by clamping the renal circulation until the alloxan in the blood was no longer active The initial hyperglycemia and the following hypoglycemia were found in both clamped and control groups[341] This effect has not been confirmed by workers outside Jimenez Diaz's group, but since their first paper they have published a series of papers confirming and extending their initial observations Thus a small dose of uranium nitrate leads to renal damage but no disturbance in carbohydrate metabolism, but the animal develops diabetes after given doses of alloxan too small to be diabetogenic in normal dogs Alloxan aggravates pancreatectomy diabetes, but not if the renal pedicles are pinched.

More recently[342] alloxan *in vitro* has been shown to reduce the oxygen uptake of the dog's kidney, though anaerobic glycolysis is not affected.

Patterson's discovery of the diabetogenic action of ascorbone[277, 343] may now be discussed at greater length It is still being exploited and is one of the most important advances in our knowledge of diabetes since the discovery of alloxan diabetes in 1943 The principal results so far obtained are as follows Ascorbone is toxic to the rat, the LD_{50} being 320 mg per kilogram, survival after a sublethal dose results in increased tolerance to subsequent doses Ascorbone is more effective in acting synergically with alloxan than is ascorbic acid The diabetogenic dose of ascorbone in rats, in divided doses over a few days, totals 1 1 g per kilogram, and isoascorbone (dehydro-D-araboascorbic acid) produces diabetes in a single dose of 1 5 g per kilogram, being less toxic than ascorbone The diabetogenic dose of alloxan for most animals including the rat is of the order of 200 mg per kilogram,[344] and the molecular weight of alloxan is 138 It thus appears to be a more powerful diabetogenic agent, molecule for molecule, than ascorbone, which has a molecular weight of 174

Patterson[344] has also shown that diketogulonic acid is not diabetogenic

[340] C Jimenez Diaz, F Grande Covián, and J C De Oya, *Nature* 158, 589 (1946)
[341] J G Villasante and C Jimenez Diaz, *Bull Inst Med Research, Univ Madrid* 3, 201 (1950)
[342] F D W Lukens, *Physiol Revs* 28, 304 (1948).
[343] J W Patterson, *Science* 111, 724 (1950)

and that D-glucoascorbone is diabetogenic in doses of about 4 g per kilogram. Glucoascorbone did not cause the nervous response (see p. 390) resulting from ascorbone and isoascorbone. The latter two compounds are derived from two antiscorbutic acids, whereas glucoascorbic acid is very slightly if at all antiscorbutic. The effect of D-ascorbone is not known. Kinsey,[78] as reported above, has shown that a similar division between the various acids exists for entry into the anterior chamber of the eye (see p. 382 above), and Patterson[246] has argued that the nervous effects of ascorbone and isoascorbone, unlike their diabetogenic effects which are shared with alloxan and D-glucoascorbone, may be related to their action as vitamins (see below). With Lazarow[247] Patterson has shown that cysteine, glutathione, and 2,3-dimercaptopropanol (BAL) protect against the diabetogenic effects of ascorbone. They argue that, like alloxan, ascorbone combines with thiols and probably causes diabetes by inactivation of essential enzymes in the beta cells. The non-reversal of ascorbone (and alloxan) diabetes by the injection of thiols after ascorbone is attributed to the relative irreversibility of the formation of the SH-ascorbone compound, and reversible oxidation of -SH to S-S compounds is therefore thought to be unlikely. *Five to six grams of ascorbic acid per kilogram is not* diabetogenic in rats.

The persistent ascorbone diabetes of rats has been shown[248] to lead, like alloxan diabetes, to cataract, the minimum duration and blood-sugar level necessary for the development of cataract being surprisingly well represented by the equation:

$$(\text{Duration in weeks} - 5.7) \times [(\text{Blood sugar in mg}/100\text{ ml}) - 225] = 1276$$

The relations among ascorbic acid, ascorbone, alloxan, and experimental diabetes have also been investigated by Italian workers. Siliprandi and Pisati[249] conclude that dialuric acid is largely converted in the rat to alloxan —a rather surprising finding in the light of the oxidation-reduction potential of the alloxan system and the work of Archibald[250] on the reverse conversion. Siliprandi[251] has also shown that the plasma ascorbic acid decreases to about half its original value after injection of alloxan into rabbits. This is consistent with his view that there is an equilibrium

$$\text{Alloxan} + \text{ascorbic acid} \rightleftharpoons \text{Ascorbone} + \text{dialuric acid}$$

and with Lloyd's observation[82] that ascorbone readily leaves the serum for the erythrocytes of defibrinated human blood *in vitro* (see p. 387 above).

Merlini[121] has observed the adrenaline-like action of ascorbone described by Patterson and Mastin[83] but denies[252] Patterson's principal observation

[247] J. W. Patterson and A. Lazarow, *J. Biol. Chem.* 186, 141 (1950).

[248] J. W. Patterson, *Am. J. Physiol.* 165, 61 (1951).

that ascorbone is diabetogenic in the rat at dose levels similar to Patterson's He also denies[110a] that ascorbic acid potentiates alloxan's diabetogenic effect and finds that the compounds interfere In the dog, combined injections led to temporary severe respiratory disturbance but not to diabetes (cf p 388).

It is tempting to speculate on the mechanisms of alloxan and ascorbone diabetes, and a connection between them may be found in the observation of Jimenez Diaz *et al* [243] discussed above, that entry of alloxan into the renal circulation is essential for alloxan diabetes Ascorbic acid is a threshold substance and is therefore at times secreted against a concentration gradient from the tubular fluid into the plasma in the kidney The concentration gradient may be a considerable one, comparable with that found in the human mammary gland (see p 399) Earlier in this chapter other secretion mechanisms involving ascorbone have been discussed It is possible that ascorbic acid is secreted in the kidney by a conversion of ascorbic acid to ascorbone, followed by a reduction of the latter to ascorbic acid Alloxan may as an oxidizing agent interfere with or destroy the reducing mechanism, with the result that ascorbone may diffuse into the blood and hence may reach the islets and give rise to diabetes This would explain the observation of Jimenez Diaz *et al* [243] and it is consistent with the apparently greater potency of alloxan than ascorbone, for a given dose of alloxan might well cause the release of several stoichiometric equivalents of ascorbone D-Glucoascorbone could act like alloxan or could oxidize ascorbic acid directly

A detailed account of the changes in the rat's kidney has been given by Gabe,[253] who has shown histochemically that some days after the administration of alloxan the ascorbic acid content of the kidney is diminished. The observation of Brückmann and Wertheimer[254] that dimethylalloxan, which is not diabetogenic, apparently leads to kidney damage hardly militates against the present hypothesis, as there are many other nephrotoxic non-diabetogenic substances

The guinea pig is resistant to alloxan diabetes at a dose level of 1 g. per kilogram [256, 257] There is a narrow margin between hyperglycemic and toxic doses affecting the kidneys and producing anemia The reduced glutathione of guinea pig blood is very high and is decreased by alloxan Charalampous and Hegsted[258] have shown that guinea pigs deficient in ascorbic acid, or starved before injection of alloxan, failed like the normal animal to develop diabetes, and—a very important observation for the present hypothesis

253 [illegible]
256 [illegible]
256 [illegible]
257 [illegible]
258 [illegible]

in relation to the observation of Jimenez Diaz *et al.*[243]—that pancreatic islet cells appear no more susceptible to alloxan than the cells of other tissues. Griffiths[259] has shown that on a diet deficient in methionine and cystine guinea pigs develop hypoglycemia immediately after alloxan. Man is now[245] thought to be susceptible to alloxan, but the high dosage apparently needed may be similarly related to his presumably relatively large renal mechanism for conservation of vitamin C. The direct or the kidney-ascorbone hypotheses of alloxan diabetes are equally consistent with the protective action of glutathione. The effects of alloxan and ascorbone on guinea pigs deficient in ascorbic acid and in organic sulfur are unknown.

The setting up of alloxan diabetes in a vitamin C-deficient animal would be a severe blow to the kidney-ascorbone hypothesis. The present data on the synergic and, according to others, antagonistic effects of ascorbic acid on alloxan in scurvy-resistant species are far from decisive in this context.

Whether the beta cells are poisoned by ascorbone, alloxan, or both, the details of the toxic action are unknown, though Lazarow's[237] ideas provide some basis for further experimental work. Lens and Neutelings[260] have *shown that cysteine and glutathione reduce insulin but that under* no conditions does ascorbic acid do so. Intravenous glutathione has been shown to alleviate corticotropin diabetes in man,[261] perhaps by providing nutritional precursors for a raised production of insulin, whereas Lazarow has shown[262] that *feeding* glutathione and cysteine increases alloxan sensitivity and that injected glutathione increased cortisone-induced glycosuria in the rat. These apparently contradictory results may be explained by species differences, but it seems that -SH compounds can under appropriate circumstances inhibit the production of insulin by the beta cells, which presumably involves or follows oxidations of thiol groups to disulfide linkages. This oxidation is possibly influenced in some way by ascorbone, apparently it is not known if incubation of pancreases with ascorbone leads to increased contents of insulin.

b. Adrenal Cortex and Hypophysis. Analytical figures for the adrenal ascorbic acid are given in Giroud *et al.*[262a] Their graph relating adrenal ascorbic acid and daily intake of ascorbic acid for the guinea pig is noteworthy (cf. Table 2), as are the extremely high levels to which adrenal ascorbate may rise and the very high daily intake needed by the guinea pig to reach an adrenal ascorbate typical of that found in the animals for which ascorbic acid is dispensable.

259 M. Griffiths, *Australian J. Exptl. Biol. Med. Sci.* 26, 339 (1948).
260 J. Lens and J. Neutelings, *Biochem. et Biophys. Acta* 4, 501 (1950).
261 [illegible] 109, 270 (1949).
262
262a

Since their work even higher figures for the rat have been reported (e g, 443 ± 28 mg per 100 g of gland, Covian[263] and see p 415)

The interpretation of these bald figures depends on the distribution of the adrenal ascorbic acid over the gland, as between medulla and cortex, and as between the zones of the cortex, and since Bourne's[264] original demonstration of adrenal ascorbic acid histochemically by reduction of silver nitrate there has been some success with this problem of distribution.[265, 266, 267]

After due allowance is made for the proportions of cortex and medulla, it may be concluded that the concentration of ascorbic acid may reach 3 to 4% of the dry weight of the mammalian adrenal cortex, and possibly more in certain cells or zones This ascorbic acid is, at least after fixation, associated with characteristic lipoid matter and the Golgi apparatus [268, 268a]

There is less information about the ascorbic acid in the pituitary Table 3 shows a figure of about 120 mg per 100 g of pituitary, remaining surprisingly constant over a range of species

The isolation of hexuronic acid from ox adrenals by Szent-Györgyi[15] was a most important though largely (and necessarily) at the time unrecognized advance in the physiology of the adrenal and of ascorbic acid, and the diminution of the normally high ascorbic acid in the adrenal in scurvy was noted by Harris and Ray (e g, ref 18) In 1944 Ratsimamanga[269] in effect confirmed Lockwood and Hartman's observation[270] that an adrenal cortical extract, lacking ascorbic acid, delays scurvy in guinea pigs This work has been further clarified by the use of the purified adrenocorticotropic hormone—corticotropin—of the anterior pituitary and of pure "cortisone," an 11-dehydro-17-ketosteroid probably very similar to the secretion or to one of the secretions of the adrenal cortex Hyman, Ragan, and Turner[271] have shown that corticotropin and cortisone prolong life and reduce hemorrhage in the guinea pig on a scorbutogenic diet, possibly by reducing tissue requirement for vitamin C, and in particular that corticotropin seems to promote corticosteroid production in the scorbutic animal Banerjee *et al*[74] have shown that the diminution of reduced ascorbic in the scorbutic adrenal, to about 10 mg per 100 g, is accompanied by an increase in the

[263] M R Covián, *Compt rend soc biol* **143**, 1252 (1949)
[264] G H Bourne, *Nature* **131**, 874 (1933)
[265] H W Deane and A Morse, *Anat Record* **100**, 127 (1948)
[266] M R Lewis, *Anat Record* **102**, 37 (1948)
[267] R Bacchus, *Am J Physiol* **163**, 326 (1950)
[268] G H Bourne, *J Roy Microscop Soc* **70**, 367 (1950)
[268a] G H Bourne, *Nature* **166**, 519 (1950)
[269] A -R Ratsimamanga, *Compt rend soc biol* **138**, 19 (1944)
[270] J. E Lockwood and F A Hartman, *Endocrinology* **17**, 501 (1933)
[271] G A Hyman, C. Ragan, and J C Turner, *Proc Soc Exptl Biol Med* **75**, 470 (1950)

in relation to the observation of Jimenez Diaz *et al.*[213]—that pancreatic islet cells appear no more susceptible to alloxan than the cells of other tissues Griffiths[259] has shown that on a diet deficient in methionine and cystine guinea pigs develop hypoglycemia immediately after alloxan Man is now[245] thought to be susceptible to alloxan, but the high dosage apparently needed may be similarly related to his presumably relatively large renal mechanism for conservation of vitamin C The direct or the kidney-ascorbone hypotheses of alloxan diabetes are equally consistent with the protective action of glutathione The effects of alloxan and ascorbone on guinea pigs deficient in ascorbic acid and in organic sulfur are unknown

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[259] M Griffiths, *Australian J Exptl. Biol Med Sci.* **26**, 339 (1948)
[260] J Lens and J Neutelings, *Biochem et Biophys Acta* **4**, 501 (1950)
[261] J W Conn, L H Louis, and M. W. Johnston, *Science* 109, 279 (1949)
[262] A Lazarow, *Proc Soc Exptl Biol Med* **74**, 702 (1950).
[262a] A Giroud, C P. Leblond, A -R Ratsimamanga, and E. Géro, *Bull soc chim biol* **20**, 1080 (1938)

ascorbate is not zero in the scorbutic animal The effect of vitamin deficiencies on adrenocortical function has recently been reviewed by Morgan.[279] Bacchus *et al* [279a] state that ascorbic acid prolongs the effect of cortisone or corticotropin in rats, possibly by lessening their inactivation or excretion

A most important observation on the physiology of the ascorbic acid in the adrenal has been that of Sayers *et al* [280] that the administration of corticotropin to an animal results in depletion of this ascorbic acid This has been repeatedly confirmed, and the adrenal ascorbic acid has been used as an indicator of adrenal cortical and hypophyseal function, particularly in animals subjected to stress, and in assay of corticotropin [281]

Sayers *et al* [282] followed up this fundamental observation with the suggestion that ascorbic acid and cholesterol may participate in the formation of the adrenal cortical hormone, for, after non-fatal hemorrhage, as the adrenal ascorbic acid decreases, the cholesterol of the adrenal and of the blood run roughly parallel with it They[282, 283] favor the hypothesis that cholesterol is the precursor of the adrenal steroids, in spite of certain chemical difficulties· it is very difficult to name another possible precursor

A large number of observations in addition to those already mentioned have been made which bear more or less directly on this point and on the relation of ascorbic acid to adrenal cortical secretion Del Castillo and Rapela[284] showed that the administration of desoxycorticosterone acetate (DOCA) induced adrenal atrophy and prevented loss of ascorbic acid from the adrenals during stress Sayers *et al* [285] showed that 15 hr after a single corticotropin injection into rats there is a rise in adrenal ascorbate. this emphasizes the dynamic state of the adrenal ascorbate

In summary,[286, 286a] adrenal ascorbate is reduced during corticotropin treatment or by a variety of stresses, including cold, infection, adrenaline injections, insulin hypoglycemia, and histamine shock Some species peculiarities are known· for example, single large doses of adrenaline or corticotropin do not reduce adrenal ascorbate in the chick,[287] although adrenaline

[279] A F Morgan, *Vitamins and Hormones* **9**, 162 (1951)

[279a] H Bacchus, N Altszuler, and M H Heiffer, *Proc Soc Exptl Biol Med* **80**, 88 (1952)

[280] G Sayers, M A Sayers, H L Lewis, and C N H Long, *Proc Soc Exptl Biol Med* **55**, 238 (1944)

[281] P L Munson and F C Koch, *Endocrinology* **39**, 76 (1946)

[282] G Sayers, M A Sayers, T -Y Liang, and C N H Long, *Endocrinology* **37**, 96 (1945).

[283] C N H. Long, *Recent Progr Hormone Research* **1**, 99 (1947)

[284] E B Del Castillo and C E Rapela, *Rev soc argentina biol* **21**, 338 (1945) [*C A* **40**, 3170 (1946)]

[285] G Sayers, M A Sayers, T -Y Liang, and C N H Long, *Endocrinology* **38**, 1 (1946)

[286] G Sayers, *Physiol Revs* **30**, 241 (1950)

[286a] G Sayers and M. A Sayers, *Recent Progr Hormone Research* **2**, 81 (1948)

[287] J. W. Jailer and N F Boas, *Endocrinology* **46**, 314 (1950)

ascorbone concentration from zero to about 3 mg per 100 g, which may indicate an increased rather than a decreased adrenal cortical function

Salmon and May[273] have shown that cortisone promotes the metabolism of tyrosine in the scorbutic monkey (see p. 431), and Bland *et al.*[274] have recently concluded that corticotropin does not hasten scurvy in the guinea pig The effect of scurvy on secretory function in the adrenal cortex has already been mentioned in connection with carbohydrate metabolism. Daughaday, Jaffe, and Williams[275] showed that in three scorbutic adults treated with vitamin C the excretion of formaldehydogenic (adrenal cortical) steroids at first decreased and then became normal or greater than normal McKee, Cobbey, and Geiman[276] found, in disagreement with Penney and Zilva,[70] that in the scorbutic guinea pig the adrenal ascorbic acid is less than that in liver, spleen or kidneys Rabinowicz and Ratsimamanga[277] have shown that deprivation of the guinea pig of vitamin C for some days leads to a reduction of the adrenal 11-ketosteroids to about 40 %. Treagar *et al.*[278] have failed to find evidence of reduced adrenocortical function in clinical human scurvy Hyman, Ragan, and Turner[271] have similarly shown that in guinea pigs with scurvy corticotropin, apart from delaying death for 8 days, led to intense hypertrophy and increasing granularity of the adrenal cortex, indicating an actively secreting gland The parallel weight gains after corticotropin and cortisone implied that cortisone-like steroids are secreted under corticotropin treatment by the scorbutic animal. Banerjee and Deb[221] have shown that adrenal cholesterol and adrenal ascorbate are both lowered in the scorbutic guinea pig. The reduction of adrenal ascorbate is about fivefold and very much greater than that of cholesterol More recently[222] they have shown that the excretion of 17-ketosteroids is somewhat reduced in scorbutic guinea pigs. The weight of the evidence (cf refs. 278*a*, 278*b*) now suggests that adrenal cortical function is normal or actually increased in scurvy (cf. p 402) Adrenal hypertrophy is a characteristic feature of vitamin C deficiency[278c] and is apparently initiated as the ascorbate:cholesterol ratio drops below 1:500 [278d]

On the other hand, ascorbic acid cannot be excluded as an essential molecule for the synthesis or secretion of cortical hormone, as adrenal

[273] R J Salmon and C. D May, *Arch Biochem Biophys* **32**, 220 (1951)

[274] M N Bland, B J Constable, L J Harris, and R E Hughes, *Biochem J.* **51**, xxxv (1952)

[275] W H Daughaday, H Jaffe, and R H Williams, *J. Clin Endocrinol* **8**, 244 (1948)

[276] [illegible]

[277] [illegible]

[278] [illegible]

[278a] [illegible]

[278b] [illegible]

[278c] [illegible]

[278d] [illegible]

ascorbone will be found at 50% oxidation to ascorbone Banerjee *et al*[75] have shown that the ascorbone in the adrenals of scorbutic guinea pigs is very much higher than that of non-scorbutic guinea pigs, and there is thus some evidence for the view that the (ascorbone) (ascorbic acid) product, and hence the concentration of monohydroascorbone, tends to remain constant throughout scurvy

In indirect refutation of Lowenstein and Zwemer's claim[293] that an ascorbic-steroid substance is to be found in the adrenal, Vogt[294] has shown that there is no increase in the ascorbic acid in adrenal vein blood during the action of corticotropin and the depletion of the adrenal ascorbic acid, although she did not examine the state of oxidation of ascorbic acid in the artery and the vein Thus adrenal ascorbic acid must be destroyed, and not liberated or secreted, during the process of its depletion The only conceivable first stage in its chemical destruction is oxidation (see below, p 445), this is probably followed by fission to oxalic acid and a C_4 compound, and probably not by hydrolysis of the lactone ring to diketogulonic acid (DKG) Vogt[294] used the method of Roe and Kuether[67], which would have included DKG, in her estimation of total ascorbic acid, and it seems therefore that in the adrenal gland the depletion of ascorbic acid proceeds through oxidation and fission to oxalic acid and a C_4 derivative If an ascorbic acid derivative is catalytic in the oxidation of cholesterol, it may be argued that the concentrations of the catalyst and precursor will determine the rate of production of the oxidation product or products and hence ultimately the secretion of the cortical hormone From this it is a natural step to propose that corticotropin is an enzyme, coenzyme, or factor in some way promoting the oxidation of ascorbic acid to ascorbone The raising of corticotropin in the adrenal cortical tissue would thus have four effects:

1 The oxidation of ascorbic acid to monohydroascorbone and/or ascorbone
2 The depletion of total ascorbic acid, in view of ascorbone's short half-life (see below, p 446)
3 The raising of the concentration of monohydroascorbone and, as a consequence,
4 Promotion of the oxidation of cholesterol and the secretion of adrenal cortical hormone

It must immediately be stated that cholesterol is not an aromatic compound and that no evidence appears to exist or to have been sought for its oxidation *in vitro* in the presence of ascorbic acid, but Warren's[295] work on the *in vitro* catalyses by ascorbic acid of the autoxidation of 3,4-benzo-

[293] B E Lowenstein and R L Zwemer, *Endocrinology* 39, 63 (1946)
[294] M Vogt, *J Physiol (London)* 107, 239 (1948)
[295] F L Warren, *Biochem J* 37, 338 (1943)

causes a reduction in the sudanophilic material (partly steroid) in the adrenal, and both substances cause adrenal hypertrophy.

The results on scorbutic animals quoted earlier in this section do not exclude ascorbic acid as an essential factor in adrenal cortical secretion The beneficial effects of corticotropin and cortisone in scurvy are not easily explained, but it may be that under the stress of scurvy more efficient use of available adrenal ascorbate is made in the presence of corticotropin supplied from external sources Cortisone or compound F should prolong life in scurvy more than the equivalent of corticotropin if the latter causes an increased consumption of ascorbic acid in the adrenal. This does not seem to have been shown, although prolonged administration of corticotropin to patients has sometimes led to scurvy-like signs readily cleared up with ascorbic acid.[288] The corticotropin content of the guinea pig hypophysis rises in vitamin C deficiency.[288a]

It has already been mentioned that a reduction in adrenal cholesterol is usually associated with a stress- or corticotropin-induced reduction of adrenal ascorbate, and that the Sayers-Long group favors the hypothesis that adrenal ascorbate in some way participates in the conversion of cholesterol into part of the secretion of the adrenal cortex *This conversion may be* assumed to consist largely of oxidation of cholesterol into compound F.[289] Pirani[289a] in his excellent review of the adrenal ascorbic acid suggests that it probably has a non-specific function related to cellular respiratory activity and metabolic rate There is nevertheless indirect evidence for a more specific function That ascorbic acid is concerned with the oxidation of the benzene ring of aromatic compounds has been shown *in vitro* (see p 448) and *in vivo* (see p. 431) Neuberger's[290] discussion of the mechanism of such oxidations *in vitro* calls for the mediation of an electrophilic (cationoid) reagent The monohydroascorbone (monodehydroascorbic acid) proposed as a participant in reactions *in vitro*, particularly by LuValle and Weissberger,[291] is discussed below (p 422), and it is there concluded that it is possibly of biological importance It may be regarded as a compound formed by dissociation of a combined form of ascorbone and ascorbic acid, analogous with alloxantin (cf p 404) It may be argued that the concen-

[illegible] of the

cen-

dro-

[288] M Stefanini and M C Rosenthal, *Proc Soc Exptl Biol Med* **75**, 806 (1950)
[288a] R C Stepto, C L Pirani, J D Fisher, and K Sutherland, *Federation Proc* **11**, 429 (1952)
[289] J W Conn, L H Louis, and S S Fajans, *Science* **113**, 713 (1951)
[289a] C L Pirani, *Metabolism* **1**, 197 (1952)
[290] A Neuberger, *Ann Rev Biochem* **18**, 243 (1949)
[291] A Weissberger and J E LuValle, *J Am Chem Soc* **66**, 700 (1944)

and DOCA increases muscle ascorbic acid markedly This bewildering array of results may imply that, as has been argued for corticotropin above, cortisone, ACE, and DOCA promote oxidation, from their effects on blood cell levels But perhaps more probable, in view of their action on the kidney, is the view that they raise blood cell levels by inhibiting the oxidation (and hence destruction) of ascorbic acid in these cells

Beck *et al*[503] have shown in man that, whereas cortisone does not increase the excretion of ascorbic acid, corticotropin does so: this can hardly be attributed to its effect on the adrenal in view of Vogt's[394] finding in the dog that the ascorbic acid in the adrenal vein does not rise above that in the artery during the action of corticotropin A valuable supplement to these studies would be an investigation of oxalate excretion and of oxalate balance in the adrenal, for if corticotropin promotes the oxidation of ascorbic acid it should increase the production of oxalic acid (see p 445)

Booker *et al*[504] have studied the effects of injection of ascorbic acid into dogs, rats, and patients on various blood concentrations Their method for erythrocyte ascorbic acid, using indophenol after precipitation without previous deoxygenation, is suspect, but perhaps their most important observation is that adrenal cortical extract and ascorbic acid together caused a 550% increase in rat adrenal ascorbic acid accompanied by a 45% decrease in adrenal cholesterol The effects of either substance separately were considerable but smaller Their observation appears to mean that, by inhibiting the production of adrenal cortical hormone (possibly by inhibition of ascorbate oxidation), ACE leads to a conservation of adrenal ascorbic acid The fall in cholesterol is less easily explained, though it may be argued that an inactive gland would not continue to accumulate cholesterol They have found some evidence that the fall in adrenal cholesterol following administration of ascorbic acid is accompanied by a rise in blood cholesterol

The suggestion that corticotropin promotes the oxidation of ascorbic acid does not necessarily lose force from the results so far obtained by administration of ascorbone to animals Most of the experiments so far done on this point have been discussed already The oral administration of ascorbone prevents scurvy but gives very low tissue levels (p 382) Kelemen, Oláh, and Majoros[505] failed to find a cortisone-like effect after giving ascorbone to intact and adrenalectomized rats, but these results cannot be adequately interpreted until more knowledge is available on the distribution and fate of ascorbone in the body of the rat Patterson[393] has not reported cortisone-like effects after injection of ascorbone

[503] J C Beck, M M English, J W Hackney, and K R Mackenzie, *J. Clin. Invest* 29, 798 (1950)

[504] W M Booker, R L Hayes, M B Sewell, and F. M Dent, *Am. J. Physiol* 166, 374 (1951)

[505] E Kelemen, F Oláh, and M Majoros, *Lancet* 261, 886 (1951).

pyrene, a true aromatic substance, is of great interest here. Tepperman[295a] has demonstrated a marked reduction in the ascorbate of dog adrenal slices incubated with purified corticotropin. Ascorbic acid augments the action of small doses of corticotropin in hypophysectomized rats.[295b]

Data on the effect of corticotropin or cortisone on the general metabolism of ascorbic acid outside the adrenal cortex are now fairly extensive Berri[296] showed that in patients with trauma the ratio of ascorbone to ascorbic acid in the urine was related to the presence of shock, varying from 0 09 to 0 69 in those without shock, and from 2 34 to 4 40 in those with shock This may be taken to support the hypothesis that corticotropin promotes the oxidation of ascorbic acid, though perhaps equally well it could be argued that the reduction of ascorbone is inhibited by the products of shock (cf. pp 417 and 452)

Booker, Hayes, and Dent[297] have shown with dogs that in the second week of therapy with ascorbic acid with or without adrenal cortical hormone the blood cell levels, unlike plasma levels, are much higher in the group receiving hormone (cf pp. 386–388), which implies a promotion of oxidation. Fortier and Skelton[299] have shown that in the glucose-treated, hypophysectomized rat, though not in the normal rat, the plasma ascorbic acid increases after a single dose of corticotropin, whereas after hypophysectomy alone there is a marked and progressive decrease of plasma ascorbic acid, evidence on the whole militating against the view that the oxidation of ascorbic acid is promoted by corticotropin.

Hayano, Dorfmann, and Yamada[300] have shown that desoxycorticosterone inhibits tyrosinase strongly and ascorbic oxidase, a plant *enzyme*, partly: this may point to a homeostatic mechanism whereby the adrenal cortical hormone opposes the promotion by corticotropin of the oxidation of ascorbic acid, though desoxycorticosterone is not the primary secretion of the adrenal cortex Booker *et al* [302] have observed the effects of adrenal cortical extract (ACE), desoxycorticosterone acetate (DOCA), and cortisone on the distribution of ascorbic acid in rats and dogs *They find that* in dogs and rats these three substances reduce the excretion and raise the plasma and cell levels of ascorbic acid. In rats all three, cortisone most, ACE least, raise ascorbic acid concentrations especially in blood cells, and also raise adrenal ascorbic acid. They reduce the concentration in the lung,

295a [illegible] (1950)
295b [illegible]
296 [illegible]
297 [illegible] 9, 14 (1950)
299 [illegible]
300 [illegible] 186, 603 (1950)
302 [illegible] Green, *Am J* [illegible]

of cortisone-like substances, for cortisone itself has a dramatic effect on rheumatoid arthritis, not necessarily without untoward side-reactions The recent work of Long and his collaborators[313] has pointed to ascorbone, rather than ascorbic acid, as a substance of possible therapeutic importance in syndromes caused by adrenal cortical hypofunction, but so far very few results of treatment with ascorbone are available, and its diabetogenic activity encourages caution in its use (cf ref 313a)

Cornforth and Long[314] now suggest that the action of cortisone in desensitizing the BCG-injected guinea pig against subsequent injection of tuberculin is due to the following chain cortisone promotes the peripheral oxidation of ascorbic acid, and the ascorbone so formed inhibits phosphoglucomutase, this leads to an accumulation of glucose-1-phosphate The evidence for this chain is largely indirect, and the general significance of glucose-1-phosphate as a factor in adrenal cortical function is unknown The work is important in drawing attention to the action of cortisone on ascorbic acid as distinct from the action of ascorbic acid in the adrenal cortex A possible mechanism for the latter has been discussed above it may be tentatively expanded to include the possibility of a reverse process in the tissues whereby cortical hormone and ascorbic acid react to give a reduction product of the former and ascorbone In man cortisone causes tissues to become more reducing, however,[314a] conditions may well differ inside and outside the cell (cf the work of Stewart *et al*[91] on blood, p. 384)

This hypothesis leads to the problem of the local relationships of cortisone and ascorbic acid to scurvy and the formation of connective tissue (discussed on pp 395 and 430)

The adrenalectomized scurvy-resistant animal shows none of the stigmata of scurvy and continues to synthesize ascorbic acid Dumm and Ralli[315] found a reduced excretion of ascorbic acid in adrenalectomized rats It is thus very unlikely that the adrenal is the sole site of synthesis of ascorbic acid (see pp 444–445)

c **Adrenaline.** Kasahara, Nishizawa, and Hirao,[316] and these same three authors with Horie,[317] claimed that simultaneous treatment with ascorbic acid was necessary for hypertension and hyperglycemia after treatment of the scorbutic guinea pig with adrenaline Blaschko, Richter, and Schlossmann[318]

[313] D A Long, A Miles, and W L M Perry, *Lancet* **260**, 1085 (1951)
[313a] G Telium, *Ann chem Dis* **11**, 119 (1952)
[314] J W Cornforth and D A Long, *Lancet* **262**, 950 (1952), **264**, 160 (1953).
[314a] G L Loxton and D Le Vay, *Nature* **171**, 524 (1953)
[315] M E Dumm and E P Ralli, *Endocrinology* **45**, 188 (1949)
[316] M Kasahara, Y Nishizawa, and S Hirao, *Klin Wochschr* **16**, 1618 (1937)
[317] M Kasahara, Y Nishizawa, H Horie, and S Hirao, *Klin Wochschr* **17**, 1260 (1938)
[318] H Blaschko, D Richter, and H Schlossmann, *J Physiol* (London) **90**, 1 (1937)

Stewart, Horn, and Robson[94] have shown that oral cortisone given to man causes a temporary decrease in plasma ascorbone, without an increase in total ascorbic acid. Corticotropin parenterally in comparable dosage causes a similar effect except that the total ascorbic acid in the plasma increases

These significant observations need to be accompanied by work on cellular ascorbone and ascorbate concentrations and their ratio for interpretation At first sight they imply that corticotropin causes the hydrogenation of ascorbone: but the direct effect of corticotropin may be masked by that of the adrenal cortical secretion that it causes

Some recent observations[306, 307] on the relations of thiol compounds to the corticotropin-adrenal-ascorbate relation are of interest Carey *et al*[308] have shown that large doses of glutathione produce toxic reactions and lower the ascorbic acid and the cholesterol in the adrenal of rats and guinea pigs It is not yet feasible to relate their effects to the possible role of ascorbic acid, but it must be remembered that —SH compounds are reducing agents for ascorbone and thus tend to lower tissue concentrations of ascorbone

In conclusion of this section the therapeutic importance of ascorbic acid in treatment of rheumatoid arthritis responding to corticotropin or cortisone may be discussed Rinehart and Mettier[310] suggested that a deficiency of ascorbic acid was part of the etiology of rheumatic fever, but no convincing evidence has yet been produced to show that rheumatic fever can be helped by dietary ascorbic acid (cf. ref 311) It has recently[311a] been claimed that cortisone administration not only prolongs life but also prevents the onset of pain in joints and maintains almost normal bone structure in scorbutic guinea pigs.

Lewin and Wassén[312] announced that almost simultaneous injections of desoxycorticosterone and ascorbic acid were beneficial in rheumatoid arthritis A large number of publications have in the main since failed to find support for this claim It may be concluded that ascorbic acid and desoxycorticosterone do not form or promote the formation and activity

306 W C Hess, L H Kyle, and P D Doolan, *Proc Soc Exptl Biol Med* **76**, 418 (1951)

307 S H Ingbar, J F Otto, and E H Kass, *Proc. Soc Exptl Biol Med* **77**, 20 (1951)

308 M M Carey, E P. Vollmer, R L Zwemer, and D L Spence, *Am J Physiol* **164**, 770 (1951)

310 J F Rinehart and S R Mettier, *Am J Path* **9**, 952 (1933)

311 B F. Massell, J E Warren, P. R. Patterson, and H J Lehmus, *New Engl J Med* **242**, 614 (1950)

311a E H Herrick, R E Mead, B Egerton, and J S Hughes, *Endocrinology* **50**, 259 (1952).

312 E Lewin and E Wassén, *Lancet* **257**, 993 (1949)

acid or its oxidation products promote this There is further discussion of adrenaline and ascorbic acid on pp 391 and 449

d. Sex and Reproduction. The increased requirement for vitamin C in pregnancy was studied extensively in the later 1930's (e g refs 326, 327, 328), and it is generally accepted that during human pregnancy and lactation (see p 400) the maternal requirement is raised by 50 or 100 mg daily above the "normal" level, whatever that may be (p 460) This increase is to be expected in view of the growth of new tissue, fetal and maternal, during pregnancy and the considerable secretion of ascorbic acid in the milk during lactation

There are differences in the metabolism of ascorbic acid between the sexes in scurvy-resistant animals such as the rat Todhunter and McMillan[116a] found that the plasma ascorbic acid was about 0 87 mg per 100 ml in male rats and 0 33 in female rats—a highly significant difference They also report that dogs show a very much smaller sex difference of about 0 05 mg per 100 ml plasma, the average male level being about 0 35 mg per 100 ml Beach, Bradshaw, and Blatherwick[330] find that, just as human diabetes is much commoner in women than men, female rats are more susceptible to alloxan than are males—a point, in view of the similarity between alloxan and ascorbone diabetes, that may be related to sex differences in the renal treatment of ascorbic acid (see p 399)

Giroud and Ratsimamanga[2] have given a summary of the course of ascorbic acid followed histochemically and chemically during the existence of the corpus luteum During the follicular phase, the follicle shows no staining with the silver nitrate reagent At the luteal phase there is precipitation of silver granules in the corpus luteum A high concentration of ascorbic acid is thus associated with the secretion of progesterone, and they tentatively classify the corpus luteum with the adrenal cortex and the interstitial cells of the testicle as needing ascorbic acid for the secretion of a steroid hormone The ascorbic acid content of the ovary is 15 to 20 mg per 100 g , the corpus luteum itself is much richer in ascorbic acid, and Table 10, condensed from Giroud and Ratsimamanga,[2] gives the changes in ascorbic acid concentration during the cycle of the corpus luteum of the cow

In the guinea pig Mouriquand and Schon[331] have shown that pregnancy lessens the effects of ascorbic acid deficiency, and it is known that the corpus luteum is not necessary for reproduction in the later stages of

[326] W Neuweiler, *Klin Wochschr* **14**, 1040 (1935)
[327] G Mouriquand, *Bull acad méd Paris* **114**, 199 (1935)
[328] A W Fleming and H N Sandford, *J Pediat* **13**, 314 (1938).
[330] E F Beach, P J Bradshaw and N R Blatherwick, *Am J Physiol* **166**, 364 (1951)
[331] G Mouriquand and J Schön, *Compt rend* **197**, 203 (1933)

obtained results *in vitro* that suggest that initially the atmospheric oxidation of adrenaline is accelerated by ascorbic acid. Daoud and El Ayyadi[319] concluded that the administration of ascorbic acid did not enhance the pressor or metabolic effects of adrenaline in the cat, and Beyer[320] in a preliminary report suggested that ascorbic acid could inhibit the action of pressor amines by assisting in their deamination. Falk[321] has proposed that adrenochrome is a catalytic hydrogen carrier in the oxidation of adrenaline and in the atmospheric oxidation of ascorbic acid

OH, H, HO, CH—CH_2—N—CH_3, HO

$\xrightarrow{+O_2}$

Adrenaline

OH, H, O, CH—CH_2—N—CH_3, O, $+H_2O_2$

$\xrightarrow{+\frac{1}{2}O_2}$

O, CHOH, CH_2, N, O, CH_3 + H_2O

Adrenaline quinone

Adrenochrome

Renevey[322] has concluded that ascorbic acid does not protect adrenaline in blood, but the synergism of ascorbic acid and adrenaline *in vivo* has been confirmed in the scorbutic guinea pig by Shimamura[323] and Freire[324] Giroud and Martinet[325] have found that the adrenaline and the ascorbic acid in the adrenal vary inversely during the course of vitamin C deficiency in the guinea pig In summary it may be stated that both ascorbic acid and adrenaline are reducing substances, both autoxidizable *in vitro* and possibly mutually catalytic in respect of their autoxidations There is evidence of a synergic action *in vivo* in the scorbutic guinea pig, but its mechanism is unknown One may speculate that the action of adrenaline depends on the process or result of its peripheral oxidation and that ascorbic

[319] K M Daoud and M A S El Ayyadi, *Biochem J.* **32**, 1424 (1938).
[320] K H. Beyer, *Am J Physiol* **133**, 214 (1941)
[321] J E Falk, *Biochem J* **44**, 369 (1949)
[322] [illegible] *Lausanne*-
[323] [illegible] (1938)]
[324] [illegible] (1941)]
[325] [illegible]

and analogous with the suggestion that the oxidation of ascorbic acid in the adrenal cortex is under the control of corticotropin

e. Thyroid. The earlier work on the reciprocal interrelations between vitamin C and the thyroid has been summarized by Drill.[339] He concluded that there was good evidence that scurvy, especially chronic scurvy, promotes hemorrhagic infiltration and hyperplasia in the thyroid Hyperthyroidism appears to increase the requirement for dietary vitamin C and to reduce its concentration in tissues in general and in the adrenal in particular

More recently Johnson, Hansen, and Lardy[342] have shown that desiccated thyroid gland and thiourea diminished the ascorbate in the plasma and tissues of the rabbit, and Rossi[343] has shown that the vitamin C content of the adrenals is lowered in hyperthyroid rabbits Chanda, McNaught, and Owen[344] reported that thyroxine decreases and thiouracil increases the ascorbate in cow's milk

The increase followed by a return to normal in adrenal ascorbic acid of the male rat under thiouracil treatment contrasts with the final decrease after thyroidectomy and is accompanied by a greater adrenal atrophy These changes in concentrations are prevented by thyroxine [335]

Thyroxine appears to be essential for "desensitization" of the guinea pig by cortisone,[345] which may involve the oxidation of ascorbic acid (see p 417) Gemmill[346] has shown that thyroxine accelerates the oxidation of ascorbic acid in the presence of plant ascorbic oxidase and has proposed a mechanism involving a free radical of thyroxine The results of his promised search for an animal copper-containing enzyme linking ascorbic acid and thyroxine in animals are eagerly awaited (cf ref 347)

3 Localization in Cells

The older, histochemical methods for "localizing" substances in cells have been applied to ascorbic acid since its first characterization, Bourne[348] in 1933 having shown that ascorbic acid may be demonstrated microscopically by "staining" with silver nitrate which is reduced to metallic silver He thus showed its high concentration in the adrenal cortex In 1935 Glick and Biskind[349] used a chemical method for showing the quantitative distribution of ascorbic acid in microtome sections of beef adrenal Cy-

[339] V A Drill, *Physiol Revs* **23**, 355 (1943)
[342] R B Johnson, R G Hansen, and H A Lardy, *Arch Biochem* **19**, 216 (1948)
[343] C A Rossi, *Boll soc ital biol sper* **24**, 1260 (1948)
[344] R Chanda, M L McNaught, and E C Owen, *Biochem J* **45**, xix (1949)
[345] R R Freedman and A S Gordon, *Proc Soc Exptl Biol Med* **75**, 729 (1950).
[346] C L Gemmill, *J Biol Chem* **192**, 749 (1951)
[347] C L Gemmill, *Federation Proc* **11**, 318 (1952)
[349] D Glick and G R Biskind, *J Biol Chem* **110**, 1 (1935)

pregnancy This is consistent with the view that ascorbic acid is involved in the synthesis of progesterone

Hoch-Ligeti and Bourne[332] have shown that the ovaries of rats show cyclic changes in concentration and histological distribution of ascorbic acid during the estrus cycle, though they did not observe cyclic changes in the ascorbic acid granules of corpora lutea. Miller and Everett[333] showed that ascorbic acid accumulates in the corpora lutea as cholesterol accumulates under the influence of pituitary luteinizing hormone, and that an excess of lactogenic hormone may decrease the concentration of ascorbic acid De Allende, de Caligaris, and Orías[334] have shown that 5 mg ascorbic acid injected into the ligatured oviduct induces abundant secretion from its glands in the toad Claesson *et al* [335] and Hokfelt[336] have shown that the intravenous injection of mare serum gonadotrophin into rabbits on the twelfth day of pregnancy causes in 3 hr a fall of about 35% in the ascorbic acid content of the interstitial gland of the ovary and a decrease of about

TABLE 10

ASCORBIC ACID IN THE CORPUS LUTEUM

Stage of corpus luteum	Ascorbic acid, mg /100 g
Young	75
Adult	103
Regressing	58

25% in the corpus luteum, with recovery in about 24 hr Chorionic gonadotropin did not affect the plasma ascorbic acid level in two women [337] There seems to be no evidence against the suggestion of Giroud and Ratsimamanga[2] that ascorbic acid is consumed during the secretion of progesterone by the gonadotropin-stimulated corpus luteum, and this proposed consumption may be related to the sex difference in plasma level found by Todhunter and McMillan [116a] Its relation to urinary excretion[191, 315, 338] (cf p 399) is not clear (see also p 397). The suggestion that gonadotropin promotes the oxidation of ascorbic acid in the corpus luteum is speculative

[332] C Hoch-Ligeti and G H Bourne, *Brit J Exptl Path* **29**, 400 (1948)

[333] D C Miller and J W Everett, *Endocrinology* **42**, 421 (1948)

[334] I L C de Allende, L S de Caligaris, and O Orías, *Rev soc argentina biol* **24**, 301 (1948)

[335] L Claesson, N Å Hillarp, B Hogberg, and B Hökfelt, *Acta Endocrinol* **2**, 249 (1949)

[336] B Hökfelt, *Acta Physiol Scand* **20**, 172 (1950)

[337] C W Lloyd, E C Hughes, M L Eva, and J Lobotsky, *J Clin Endocrinol* **9**, 268 (1949)

[338] E Bencze and M Csillag, *Magyar Nöorvosok Lapja*, **13**, 232 (1950) [*C A* **45**, 1218 (1951)].

ascorbic acid and Géro and Le Gallic[339] have found evidence for two stages in the Cu-catalysed autoxidation

The oxidation of ascorbic acid by other non-biological systems has been studied in connection with the estimation of the reduced acid (see p 376), with the preservation of reduced ascorbic acid in food, and with the preparation of ascorbone (p 376) The biological importance of monohydroascorbone is still in question Huszák[340] showed that flavones greatly accelerated the oxidation of ascorbic acid by peroxidase and hydrogen peroxide, and Bezssonoff and Woloszyn[341] produced some evidence for the existence of a dimeric form of monohydroascorbone, analogous with alloxantin (cf ref 250) Some support for the view of Weissberger and LuValle[291] that one electron is involved in the initial oxidation of the ascorbate comes from the presence of copper in ascorbic oxidase The mechanism of oxidase action is discussed below

Bevilacqua[342] found no evidence that monohydroascorbone is formed during the oxidation of ascorbic acid with irradiated methylene blue, and it is doubtful if her method would detect it The relevance of Wahren's data to the existence of monohydroascorbone has already been discussed on p 377

Definite chemical evidence for the existence of monohydroascorbone in solution or in solid form is not available It is at present a largely hypothetical intermediate for a variety of oxidations in which ascorbic acid appears to intervene, *in vivo* and *in vitro*, and more work on it is required

The study of animal systems has not so far completed the chain of oxidative and reductive reactions involving ascorbic acid, though throughout this chapter will be found references to rather special oxidations for which the presence of ascorbic acid appears to be essential There is no conclusive evidence that ascorbic acid is in animals a general respiratory catalyst comparable with the cytochromes or the pyridine nucleotides Harrison[343] demonstrated that ascorbic acid increased the oxygen uptake *in vitro* of liver tissue from scorbutic guinea pigs by an amount some six times as great as was accounted for by the equivalent of the added ascorbic acid This finding has been confirmed in effect by Quastel and Wheatley.[344] Snow and Zilva[345] found a similar increased uptake of oxygen in liver slices from non-scorbutic guinea pigs on restricted intakes of vitamin C

338 V P Calkins and H A Mattill, *J Am Chem Soc* **66**, 239 (1944)
339 E Géro and P Le Gallic, *Bull soc chim biol* **34**, 548 (1952).
340 S Huszák, *Z physiol Chem* **247**, 239 (1937)
341 M N Bezssonoff and M Woloszyn, *Bull soc chim biol* **20**, 93 (1938).
342 L Bevilacqua, *Boll soc ital biol sper* **25**, 349 (1949)
343 D C Harrison, *Biochem J* **27**, 1501 (1933)
344 J H Quastel and A H M Wheatley, *Biochem J.* **28**, 1014 (1934)
345 G A Snow and S S Zilva, *Biochem J* **35**, 787 (1941)

tochemical methods have been used in the study of ascorbic acid in embryos[351, 352] Bourne (e g , ref 268) has concluded that ascorbic acid is associated with the Golgi apparatus. There appear to have been no chemical studies based on modern methods of separating cellular elements Reiner[352a] has argued that the histochemical method of locating ascorbate is suspect

4 Enzymic and Metabolic Aspects

a. The Redox System of Ascorbic Acid and Ascorbone. (1) *General Aspects* Szent-Györgyi[12, 13] showed that his hexuronic acid was a carbohydrate-like substance with very powerful reducing properties, and in 1933 Herbert *et al*[16] expounded the chemistry of its oxidation. They concluded that it is probably best regarded as the addition of two hydroxyl groups to the double bond of ascorbic acid, though ascorbone is usually regarded as being a diketo compound

The relation between chemical and "biological" processes leading to the oxidation of ascorbic acid has been of great interest since its isolation, and various substances promoting the oxidation of ascorbic acid have been examined Barron, deMeio, and Klemperer[354] examined the action of copper as a model for the oxidation of ascorbic acid and the so-called irreversible autoxidation of ascorbic acid at pH's equal to or greater than 7.4

The copper-catalyzed oxidation of ascorbic acid has been studied carefully because of its relation to the copper-containing ascorbic oxidase of plants (see p 452) and because of the need for protection of ascorbic acid against atmospheric oxidation during analytical procedures. Metaphosphoric acid and sodium chloride are the extracting agents of choice, as they inhibit oxidation (cf refs 355, 356, and 357).

The mechanism of the copper-catalyzed oxidation has been partly worked out by Weissberger and LuValle.[291] Regarding the monovalent ascorbate anion as the substrate, they consider Cu^{++} as an electron acceptor, which can coordinate with the substrate before an electron shift, giving an ascorbic semiquinone, takes place Cu^{+}, which coordinates less readily, is released and oxidized by oxygen. The possible reactions of the semiquinone, presumably identical with the monodehydroascorbic acid (monohydroascorbone) of other writers will be discussed below Calkins and Mattill[358] have also discussed the mechanism of the oxidation of

351 [illegible]
352 [illegible]
352a [illegible]
354 [illegible] (1936)
355 [illegible] (1937)
356 [illegible]
357 [illegible]

with oxygen Borsook *et al* observed that ascorbone is rapidly reduced in the tissues other than blood and concluded that glutathione was probably the principal reducing agent

Stotz *et al*[373] observed a slow aerobic oxidation of ascorbic acid added to guinea pig liver brei and concluded that the cytochrome system was chiefly responsible They could find no evidence for an oxidase or a copper-containing system specific for ascorbic acid and concluded that ascorbic acid was not an essential intermediate in reversible oxidation of the glutathione Keilin and Hartree[374] have shown that ascorbic acid is rapidly oxidized by cytochrome oxidase plus cytochrome c Cavallini[375] has shown that the ascorbic acid content (indophenol reduction) of macerated guinea pig liver falls after 2 hr of incubation An immediate and persistent decrease after the addition of monoiodoacetic acid at the beginning of incubation was attributed to blocking of reduction of ascorbone by thiol groups Subsequent addition of potassium cyanide arrests both oxidative and reductive metabolism of ascorbic acid Watts[376] has shown that the oxidation of ascorbic acid in brain homogenates is inhibited by a variety of local anesthetics, possibly by a block at the cytochrome c-cytochrome oxidase level Giri[377] has suggested that the factors causing oxidation of ascorbic acid are mostly in the nuclear fraction of rat liver homogenates and that inhibitory factors are present in all fractions

It may be added that Lenggenhager[378] has shown that whereas normal brain, liver, and kidney have a strong reducing action, shown by their reduction of indigodisulfonic acid, tissues exposed to 6 atm of pure oxygen rapidly lost this reducing power Oxygen poisoning *in vivo* appeared to result in acute brain damage Measurements of ascorbic acid were not made, but they would perhaps have thrown light on the mechanism of poisoning of the brain by oxygen

Some aspects of biological systems reducing ascorbone have been touched upon immediately above, both the inhibition of oxidation of ascorbic acid and the reduction of ascorbone have been associated with thiol compounds by a variety of workers, though the two processes are distinct in mechanism

In 1936 Hopkins and Morgan[379] showed that a plant enzyme catalyzed the reduction of ascorbone by glutathione, but no such enzyme could be

[373] L Stotz C J Harrer, M O Schultze, and C G King, *J Biol Chem* 122, 407 (1937)
[374] D Keilin and E F Hartree, *Proc Roy Soc (London)* B125, 171 (1938)
[375] D Cavallini, *Arch sci biol (Napoli)* 32, 107 (1948)
[376] D T Watts, *J Pharmacol* 96, 325 (1949)
[377] K V Giri, *Nature* 166, 441 (1950)
[378] K Lenggenhager, *Z ges exptl Med* 116, 353 (1950)
[379] F G Hopkins and E T Morgan, *Biochem J* 30, 1446 (1936)

when L-ascorbic, D-glucoascorbic, or reductinic acid was added, and cast doubt on the relation of the phenomenon to scurvy

In general, most animal tissue preparations inhibit rather than promote the oxidation of ascorbic acid in appropriate buffer solutions[366] De Caro and Giani[368] showed that aqueous, ethanolic, or trichloroacetic acid extracts of normal or hypovitaminous guinea pigs inhibit the *in vitro* oxidation of ascorbic acid, as does tissue *brei* They concluded that glutathione and cysteine were probably responsible for the inhibition by tissues The highest inhibition was shown by the adrenals, but their method of estimation involved precipitation with trichloroacetic acid and possibly oxidation of ascorbic acid during this process.

Kellie and Zilva[369] examined the oxidation of ascorbic acid by a variety of systems, biological and inorganic, and found that washed leucocytes were exceptional in that they alone did not inhibit the autoxidation of ascorbic acid in distilled water or water containing added iron or copper They noted the oxidation of ascorbic acid by hemolyzed red blood cells and the lack of oxidation by hemolysis of red blood cells previously treated with carbon monoxide They concluded that there was no evidence that ascorbic acid is reversibly oxidized in the organism, partly from their finding that administered ascorbone is excreted as ascorbic acid in the urine Both normal and scorbutic guinea pig tissue extracts inhibited the oxidation of ascorbic acid Later work has shown that the oxidation of ascorbic acid in the organism certainly takes place

Barron, Barron, and Klemperer[370] divided biological fluids and extracts into two groups, those that protected ascorbic acid against oxidation (largely animal) and those that did not (largely vegetable)

Borsook *et al*[371] found, unlike Barron *et al*,[370] that ascorbic acid remains reduced longer in whole blood than in plasma, and they concluded that the erythrocytes of beef, cat, dog, man, pig, rat, and sheep are almost if not quite impermeable to added ascorbic acid (see p 386) They also concluded that human blood contains no mechanism for reducing ascorbone or retarding its irreversible change This erroneous conclusion resulted from their use of the indophenol method on plasma only and their failure to note the movement of ascorbone into red cells and its reduction therein (see p 387 above) It is possible that it is this removal of ascorbone by red cells that inhibits the autoxidation of ascorbic acid in whole blood by reducing the concentration of the free radical form that may react directly

[366] C A Mawson, *Biochem J* **29**, 569 (1935)
[368] L de Caro and M Giani, *Z physiol Chem* **228**, 13 (1934)
[369] A E Kellie and S S Zilva, *Biochem J* **29**, 1028 (1935)
[370] E S G Barron, A G Barron, and F Klemperer, *J. Biol Chem* **116**, 563 (1936)
[371] H Borsook, H W Davenport, C E P. Jeffreys, and R C Warner, *J Biol Chem* **117**, 237 (1937)

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[374] D Keilin and E F Hartree, *Proc Roy Soc (London)* **B125**, 171 (1938)

[375] D Cavallini, *Arch sci biol (Napoli)* **32**, 107 (1948)

[376] D T Watts, *J Pharmacol* **96**, 325 (1949)

[377] K V Giri, *Nature* **166**, 441 (1950)

[378] K Lenggenhager, *Z ges exptl Med* **116**, 353 (1950)

[379] F G Hopkins and E T Morgan, *Biochem J* **30**, 1446 (1936)

found in hepatic tissue, in which glutathione and ascorbic acid seemed to be independently oxidized Schultze, Stotz, and King[380] showed that ascorbone was rapidly reduced by animal tissues including erythrocytes, but not by serum Heat-coagulated liver reduced ascorbone more effectively than fresh liver, and iodoacetate, arsenite, and alloxan destroyed the reducing effect of tissues In the presence of a large excess of ascorbic acid the reduction of ascorbone by tissues was inappreciable, and the authors concluded that the reduction of ascorbone by animal tissues depended largely on the reversible reaction between ascorbone and thiol components of the tissues.



$$A + 2RSH \rightleftharpoons AH_2 + RS{-}SR$$

Other work on the reduction of ascorbone by blood has already been discussed Gol'dshtein and Vol'kenzon[381] have confirmed the conclusion of Schultze *et al* that some animal tissues reduce ascorbone. Very little further work on the mechanism of the reduction of ascorbate by animal tissues has been done, though recent results already mentioned on the eye, the nervous system, and the erythrocyte point to the importance of such work. Cavallini and Bonetti[382] have produced some evidence that ascorbate is involved in the oxidation of pyruvic acid *in vivo* Matusis[383] has assessed the relative reducing powers of human tissues with respect to ascorbone and has shown a decrease in some pathological states in various species Rall and Lehninger[384] have demonstrated a triphosphopyridine-nucleotide-specific glutathione reductase in rat liver The reaction



$$TPNH_2 + GSSG \rightarrow 2GSH + TPN$$

could not be shown to be reversible This reaction may be linked with the reaction·



$$A + 2GSH \rightleftharpoons GSSG + AH_2$$

The latter, as an enzymic reaction, has not yet been demonstrated in animal tissues, however, as has already been stated (cf ref. 385) Further work on the reduction of ascorbone by plant and bacterial systems is given on pp. 454 and 470

The reduction of ascorbone by the animal has been shown by feeding it to guinea pigs and to man Demole[387] showed ascorbone to be antiscorbutic in the former, and this has been confirmed, though Roe and Barnum[77] and Fox and Levy[77a] showed that tissue concentrations of ascorbic acid were

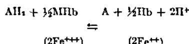
[380] M O Schultze, E Stotz, and C G King, *J Biol Chem* **122**, 395 (1937–1938)
[381] [illegible] 446 (1938)
[382] [illegible] 6)
[383] [illegible]
[384] [illegible]
[386] [illegible]
[387] V. Demole, [illegible]

very much lower on an ascorbone than an ascorbic acid diet, an observation rather difficult to correlate with the affinity and reducing power of the erythrocyte, the nervous system, and, to a smaller extent, the eye for ascorbic acid It may be linked with the observations of Banerjee *et al*[73] and of others that in guinea pigs deprived of ascorbic acid the absolute and relative concentrations of ascorbone are raised, whereas those of ascorbic acid, as expected, are very much reduced That there may be a mechanism maintaining the (ascorbic acid) × (ascorbone) product in the tissues of the organism (cf p 413) has been mentioned above

Todhunter, McMillan, and Ehmke[189] have stated from measurements of ascorbic acid in serum and urine that ascorbone is utilized well in man, though no estimations were made in tissues, such as leucocytes, in spite of the indications of the above work[77, 77a] Ritter, Cohen, and Rubin[190] have shown, on the perhaps inadequate criterion of the excretion after a dose of approximately 300 mg, that ascorbic acid and ascorbone are equivalent in physiological availability and, incidentally, equivalent to palmitoyl-L-ascorbic acid

(2) *Hemoglobin* The reduction of methemoglobin (MHb) in the mammal normally appears to proceed spontaneously Lian, Frumusan, and Sassier[191] and Deeny, Murdock, and Rogan[192] found that ascorbic acid reduced

forward reduction, and the reaction in erythrocytes *in vitro* has been worked on by Vestling[193a]

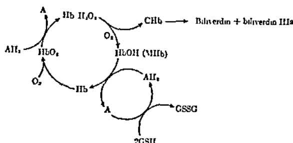


$$AH_2 + \tfrac{1}{2}MHb \underset{}{\leftrightharpoons} A + \tfrac{1}{2}Hb + 2H^+$$
$$(2Fe^{+++}) \qquad (2Fe^{++})$$

The reaction is about 95% complete towards the right-hand side This was confirmed by Gibson,[194] who showed that the reaction was in effect bimolecular, he also concluded that ascorbic acid has no place in therapy of methemoglobinemia unless the normal enzyme systems are deficient The mechanism of methemoglobin reduction *in vivo* by ascorbate is not clear: as ascorbate, if it enters the red cell at all, does so with extreme slowness (see p 424)

[189] E N Todhunter, T J McMillan, and D A Ehmke, *J Nutrition* **42**, 297 (1950)
[190] E de Ritter, N Cohen, and S. H Rubin, *Science* **113**, 628 (1951)
[191] C Lian, P Frumusan, and Sassier, *Bull mém soc méd hôp Paris* **55**, 1194 (1939).
[192] J Deeny, E T Murdock, and J J Rogan, *Brit Med J* **1**, 721 (1943)
[193] V K St G Breakey, Q H Gibson, and D C Harrison, *Lancet* **260**, 935 (1951).
[193a] C S Vestling, *J Biol Chem* **143**, 439 (1942).
[194] Q H Gibson, *Biochem J* **37**, 615 (1943)

Most of the *in vitro* work on hemoglobin and ascorbic acid has been concerned with the oxidation of hemoglobin. Lemberg, Legge, and Lockwood in 1938[395] published a preliminary paper on the "coupled oxidation" of hemoglobin and ascorbic acid, in which the prosthetic group of hemoglobin was oxidized This paper was followed by some half-dozen others on the same subject (e g , ref. 395*a*) Lemberg and his school concluded that oxyhemoglobin (HbO_2) and ascorbic acid react directly, without the mediation of free hydrogen peroxide formed in the autoxidation of ascorbic acid, to give choleglobin (CHb), with a characteristic absorption band at 628 to 630 mμ Lemberg regarded these reactions as being of physiological



importance for bile pigment formation, though he regards the non-entry of ascorbic acid into erythrocytes and an inhibiting factor in their stroma as largely preventing the initial reaction between ascorbic acid and oxyhemoglobin Others have worked on this subject. Takeya[396] has confirmed and extended Lemberg's work Gardikas, Kench, and Wilkinson[397] concluded that early degradative changes in oxyhemoglobin did not take place in the circulating erythrocyte They found[398] that the non-antiscorbutic D-xyloascorbic acid could replace L-xyloascorbic acid in the reaction with oxyhemoglobin

Foulkes and Lemberg[399] have confirmed the existence of a factor in duck erythrocyte stromata inhibiting the ascorbic acid-oxyhemoglobin reaction The whole subject has recently been reviewed [400]

Falk[321] and Albert and Falk[401] have recently examined the activity of cytochrome c and methemoglobin in promoting the oxidation of ascorbic acid by intermediate oxidation of various cyclic compounds

[395] R Lemberg, J W Legge, and W H Lockwood, *Nature* **142**, 148 (1938)
[395a] R Lemberg, J W Legge, and W H Lockwood, *Biochem J* **35**, 339 (1941)
[396] D Takeya, *J Japan Biochem Soc* **21**, 134 (1949) [*C A* **45**, 1179i (1951)]
[397] C. Gardikas, J E Kench, and J F Wilkinson, *Biochem J.* **46**, 85 (1950)
[398] M E Kench, C Gardikas, and J F Wilkinson, *Biochem J* **47**, 129 (1950)
[399] E C Foulkes and R Lemberg, *Proc Roy Soc (London)* **B136**, 435 (1949)
[400] R. Lemberg and J W Legge, Hematin Compounds and Bile Pigments, Interscience Publishers, New York, 1949
[401] A Albert and J E Falk, *Biochem J* **44**, 129 (1949)

b. Hyaluronidase. Ascorbic acid can under certain conditions *in vitro* cause depolymerization of organic substances. Thannhauser, Reichel, and Grattan[402] found that it had dephosphorylating activity, and Robertson, Ropes, and Bauer[403] showed that various ascorbic acids in fairly low concentrations in the presence of hydrogen peroxide or of copper and air had the power of catalyzing a reduction of viscosity of many mucins and polysaccharides. Ascorbone and equimolar mixtures of ascorbone and ascorbic acid did not have the effect of ascorbic acid and hydrogen peroxide. They urged the possible physiological importance of these supposed depolymerizations, which were not accompanied by the liberation of reducing sugars. They later[404] found that ascorbic acid-hydrogen peroxide reduces the viscosity of collagen *in vitro*. Kindred observations have been made by Madinaveitia and Quibell,[405] by McLean and Hale,[406] by Deuel,[407] by Kertesz,[408] who demonstrated the depolymerization of pectin by hydrogen peroxide in the absence of ascorbic acid, and by Daubenmerkl,[409] who used extremely high concentrations.

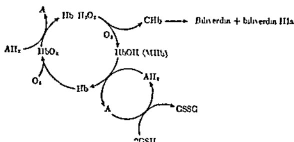
The effect of ascorbic acid on polymerized substances is to be contrasted with its effect on depolymerizing enzymes: ascorbic acid and reductone reversibly inhibit β-amylase.[410] The inhibition is reversed by oxidation and by thiols, and Hanes discusses mechanisms by which ascorbic acid and thiols acting in opposition might control enzyme systems (see also ref. 411).

Glucuronidase is inhibited by ascorbic acid[412] and by saccharic acid.[413,414] Both acids are 1,4-lactones, which Levvy[415] concludes are inhibitors.

Although it seems that ascorbic acid may control the action of hyaluronidase in some way in the synthesis of collagen, the details of the relationship are very far from clear; the work of Burns, Burch, and King[132] makes it unlikely that ascorbic acid is a component of collagen. Meyer[416] proposes that the young fibroblast secretes hyaluronic acid, a precursor of collagen, and a chondroitin sulfate. By local acidification in the immediate

[402] S. J. Thannhauser, M. Reichel, and J. F. Grattan, *Biochem. J.* **32**, 1163 (1938).
[403] W. van B. Robertson, M. W. Ropes, and W. Bauer, *Biochem. J.* **35**, 903 (1941).
[404] W. van B. Robertson, M. W. Ropes, and W. Bauer, *Proc. Soc. Exptl. Biol. Med.* **49**, 697 (1942).
[405] J. Madinaveitia and T. H. H. Quibell, *Biochem. J.* **35**, 453 (1941).
[406] D. McLean and C. W. Hale, *Biochem. J.* **35**, 159 (1941).
[407] H. Deuel, *Helv. Chim. Acta* **26**, 2002 (1943).
[408] Z. I. Kertesz, *Plant Physiol.* **18**, 308 (1943).
[409] W. Daubenmerkl, *Acta Pharmacol. Toxicol.* **7**, 153 (1951).
[410] C. S. Hanes, *Biochem. J.* **29**, 2588 (1935).
[411] P. Seshagiri Rao and K. V. Giri, *Proc. Indian Acad. Sci.* **28**, 71 (1948).
[412] B. Becker and J. S. Friedenwald, *Arch. Biochem.* **22**, 101 (1949).
[413] M. C. Karunairatnam and G. A. Levvy, *Biochem. J.* **44**, 599 (1949).
[414] B. Spencer and R. T. Williams, *Biochem. J.* **48**, 537 (1951).
[415] G. A. Levvy, *Biochem. J.* **50**, xv (1952).
[416] K. Meyer, *Physiol. Revs.* **27**, 335 (1947).

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[397] C Gardikas, J E Kench, and J F Wilkinson, *Biochem J* **46**, 85 (1950)
[398] M E Kench, C Gardikas, and J F Wilkinson, *Biochem J* **47**, 129 (1950)
[399] E C Foulkes and R Lemberg, *Proc Roy Soc (London)* **B136**, 435 (1949)
[400] R Lemberg and J W Legge, Hematin Compounds and Bile Pigments, Interscience Publishers, New York, 1949
[401] A Albert and J E. Falk, *Biochem J* **44**, 129 (1949).

by the fibroblast, cortisone appears to prevent proliferation of fibroblasts.[283, 425] Cortisone can also promote tyrosine oxidation in scorbutic guinea pigs; this fits in with the effects of corticotropin in lowering the serum hyaluronidase inhibitor and in promoting formation of pigment in a single human subject,[426] the implication being that ascorbic acid promotes the oxidation of tyrosine via homogentisic acid, while corticotropin, cortisone and possibly folic acid promote its oxidation to 3,4-dihydroxyphenylalanine (dopa) and to some extent ultimately melanin Tyrosine or, alternatively, dihydroxyphenylalanine may inhibit connective tissue formation The possible combinations of activities are numerous, and a very wide field is open for speculation and investigation

c. Tyrosine. Since 1939 Sealock and his collaborators have carried out a sustained attack on the problem of the relation of ascorbic acid to tyrosine metabolism, and Sealock's untimely death in 1951 came just as the problem was nearing solution Sealock and Silberstein[427] showed that tyrosine metabolism is defective in deficiency of ascorbic acid in the guinea pig, and Levine, Gordon, and Marples[428] observed that premature infants fed on cow's milk, a diet high in protein and low in ascorbic acid, excreted an abnormally large quantity of "tyrosyl" derivatives in the urine as long as ascorbic acid was withheld Rogers and Gardner[429] found similar phenomena in scorbutic adults receiving 20 g of tyrosine per day

The elucidation of the molecular pattern of this disorder has been left to those outside the clinical field working on animals and animal tissues *in vitro* In 1946 Basinski and Sealock[430] showed that dietary ascorbic acid had no effect on the excretion by scorbutic guinea pigs of keto acids and phenolic substances after the ingestion of eight compounds closely related to L-tyrosine They confirmed the earlier observation that only L-phenylalanine, phenylpyruvic acid, and L-tyrosine have metabolisms, as investigated in this way, depending on ascorbic acid Zilva has made several contributions to this field of the metabolism of ascorbic acid Painter and he[431] in 1947 showed that the scorbutic guinea pig excretes substances which induce methemoglobinemia and substances which oxidize potassium iodide, but that homogentisic acid is not excreted At the same time Sealock and Lan[432] showed that kidney slices from scorbutic animals have a very

[425] G H Bourne, *Intern. Z Vitaminforsch* **24**, 318 (1952)
[426] R G Sprague, M H Power, H L Mason, A Albert, D R. Mathieson, P. S. Hench, E C Kendall, C H Slocumb, and H F Polley, *Arch Internal. Med.* **85**, 199 (1950).
[427] R R Sealock and H E Silberstein, *J Biol Chem.* **135**, 251 (1940)
[428] S Z Levine, H H Gordon, and E Marples, *J Clin Invest* **20**, 209 (1941)
[429] W F Rogers and F. H Gardner, *J Lab Clin Med* **34**, 1491 (1949).
[430] D H Basinski and R R Sealock, *J Biol Chem* **166**, 7 (1946)
[431] H A Painter and S S Zilva, *Biochem J* **41**, 511 (1947)
[432] R R Sealock and T. H. Lan, *J. Biol Chem* **167**, 689 (1947).

neighborhood of the cells—possibly ascorbic acid promotes this by catalysis of the oxidation of thiol sulfur to sulfate—denaturation of the native soluble collagen gives insoluble fibers on the surface of which lies a layer of polysaccharides With aging of the fibers the polysaccharide layer becomes thinner (this is strongly supported by Bradfield and Kodicek[150]) and hyaluronic acid is replaced more and more by chondroitin sulfate It is possible that ascorbic acid is needed for this removal of hyaluronate, presumably by hydrolysis, and the possible role of ascorbic acid in the formation of organic sulfates has already been discussed.

The effect of ascorbic acid on hyaluronidase seems to be in some doubt Parrot and Fasquelle[417] report that catechol with ascorbic acid reverses the diffusing action of crude hyaluronidase They are supported by Reppert, Donegan, and Hines,[418] but Santamaria and Castellani[419] find ascorbate has no effect on illuminated hyaluronidase. Schack, Whitney, and Freeman[420] have found that the serum non-specific inhibitor of hyaluronidase is increased in scurvy in the guinea pig Quinonoid compounds inhibit the enzyme *in vitro*[420a] and may be responsible. Dosage with tyrosine hastens the increase in the inhibitor and promotes methemoglobinemia

Adrenal cortical hormones[421] *in vivo* and corticotropin inhibit hyaluronidase, possibly by a general action on thiol groups,[422] although corticotropin decreases serum hyaluronidase inhibitor [423] Layton[424] has shown that in rat tissues cortisone inhibits the synthesis of chondroitin sulfate and causes a large negative sulfur balance

It has already been noted that cortisone inhibits wound-healing in the rabbit and at very high dose levels in the guinea pig Thus, the results on cortisone, if hyaluronidase is concerned in wound-healing, suggest that its activity, not its inhibition, is part of this process The extreme metabolic inertia of collagen makes it unlikely that its integrity depends on a maintained inhibition of an enzyme system It is possible that hyaluronidase is concerned in the conversion of a precursor to collagen; ascorbic acid may promote this conversion, or it may, possibly in addition, promote chondroitin sulfate synthesis for replacement of hyaluronic acid as suggested above. Whereas scurvy appears to prevent the formation of true collagen

417 J L. Parrot and R. Fasquelle, *Compt rend soc biol* **143**, 932 (1949)

418 E Reppert, J Donegan, and L E. Hines, *Proc Soc Exptl Biol Med.* **77**, 318 (1951)

[illegible] 26, 66 (1950)

[illegible] (1950)

422 G E Anderson, [illegible] *Proc Soc* [illegible] 1951)

423 [illegible] (1950)

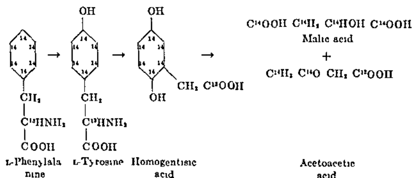
424 [illegible]

observations[29a] on the catalysis of the autoxidation of 3,4-benzopyrene are relevant here (see p 448)

Woodruff *et al* [436] showed that pteroylglutamic acid and its triglutamyl derivative, as well as ascorbic acid, could prevent hydroxyphenyluria, though not scurvy, in guinea pigs fed on scorbutigenic diets Rodney, Swendseid, and Swanson[436a] followed up their earlier work and that of Woodruff *et al* [436] by confirming that there is reduced oxidation of tyrosine in rat liver tissue after deficiency of pteroylglutamic acid, in their deficient (rat) preparations the presence of ascorbic acid had no effect on the oxidation of tyrosine

Sealock and Goodland[437] showed that cell-free homogenates of guinea pig liver oxidize L-tyrosine with two molecules of oxygen per molecule of tyrosine Dialysis reversibly inactivated the preparation That the locus of action of ascorbic acid in the metabolism of tyrosine precedes the production of 2,5-dihydroxyphenyl acids was confirmed by Clegg and Sealock,[437a] who showed that kidney extracts of normal and scorbutic guinea pigs metabolized this substance equally well

Further progress depended on fundamental knowledge of tyrosine's metabolism, and in 1949 Lerner,[439] using phenylalanine with C^{14} in the benzene ring carbons and C^{13} in the α-carbon, was able to show that in rat liver the chain of oxidation is as follows

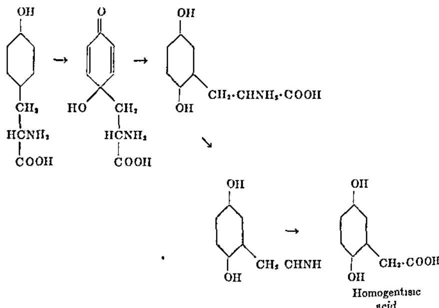


Lerner and Fitzpatrick[440] state that melanin formation cannot be initiated by the action of tyrosinase on tyrosine or 3,4-dihydroxyphenylalanine while ascorbate is present The tyrosinase-catalyzed reactions are

[436] C W Woodruff, M E Cherrington, A K Stockell, and W J Darby, *J. Biol Chem* **178**, 861 (1949)

much reduced power of oxidizing amino acids, and Rodney, Swendseid, and Swanson[433] demonstrated a lowered tyrosine metabolism in rat liver homogenates in deficiency of pteroylglutamic acid. Clegg's[434] work on kidney extracts pointed to a stage between tyrosine and 2,5-dihydroxyphenyl compounds as depending on ascorbic acid.

Feeding experiments with 2,5-dihydroxyphenylalanine[435] supported, but did not prove, the scheme of tyrosine metabolism given below which is now known not to be the main pathway:



Neuberger in 1949[290] reviewed the possible reactions in tyrosine metabolism and suggested that the oxidation of the benzene ring generally involves *attack by a cationoid (electrophilic) reagent at the ortho and para positions* relative to the first hydroxyl. The carbon chain may facilitate the attack at the *para* position, or steric factors may promote this attack. He proposed that the negative phenoxide (phenolate) ion loses two electrons to the cationoid reagent, to form a carbonium cation which with hydroxyl gives eventually a *p*-quinol. He makes the important point that similar mechanisms, involving migration of carbon chains after the introduction of oxygen, have recently been observed in "pure" organic chemistry. Neuberger did not suggest that a derivative of ascorbic acid was the cationoid reagent, but the free radical monohydroascorbone might act thus. Warren's

433 [illegible] *J. Biol. Chem.* 168, 395 (1947).
434 [illegible]
435 [illegible]

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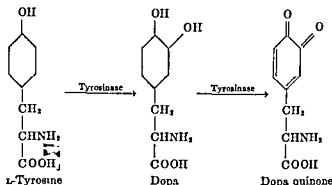
The metabolism of tyrosine and phenylalanine in premature infants in relation to ascorbic acid has been further studied by Woolf and Edmunds,[447] and La Du and Greenberg[448] have confirmed Lerner's[439] suggested mechanism for the oxidation of tyrosine Schepartz[449] has also concluded that deamination, probably involving α-ketoglutarate, precedes oxidation of the aromatic ring Fishberg's[450] claim that benzoquinone acetic acid (the quinone of homogentisic acid) occurs in scurvy rather militates against the hypothesis that ascorbic acid assists in the introduction of the second aromatic hydroxyl, but Consden *et al* [451] have shown that homogentisic acid and its quinone are absent from the urine of rheumatic fever patients, scorbutic guinea pigs, and rats with experimental methemoglobinemia

An important recent paper on this subject is that of Knox and LeMay-Knox [452] They have shown for the first time that α-ketoglutarate and ascorbate are the major cofactors in the oxidation of tyrosine in enzyme systems prepared from rat liver D-Araboascorbate can replace L-xyloascorbate The ascorbates catalyze the oxidation of the deaminated 4-hydroxyphenylpyruvate to 2,5-dihydroxyphenylpyruvate and the stages between tyrosine and homogentisic acid are thus known in some detail. In acetone-powder preparations of liver, hydroquinone is as effective a catalyst as the ascorbic acids Some other aromatic compounds are less effective [452a]

Two posthumous papers from Sealock's laboratory[225a, 234] independently show that a very similar system operates in the liver of the guinea pig The role of ascorbic acid in tyrosine metabolism is the only section of the vitamin's activity in which the molecular processes that depend upon it are fairly clearly understood It seems very unlikely that the whole of the activity of ascorbic acid depends on this role, though an extension of the

[445] K G Rienits, *J Biol Chem* 182, 11 (1950)
[445a] B Schepartz and E M Nadel, *Federation Proc* 11, 425 (1952)
[446] H A Painter and S S Zilva, *Biochem J* 46, 542 (1950).
[447] L I Woolf and M E Edmunds, *Biochem J*, 47, 630 (1950)
[448] B N La Du and D M Greenberg, *J Biol Chem* 190, 245 (1951)
[449] B Schepartz, *J Biol Chem* 193, 293 (1951)
[450] E H Fishberg, *J Biol Chem* 172, 155 (1948)
[451] R Consden, H A W Forbes, L. E Glynn, and W M Stanier, *Biochem. J.* 50, 274 (1951)
[452] W E Knox and M LeMay Knox, *Biochem J* 49, 686 (1951).
[452a] B N La Du and D M Greenberg, *Science* 117, 111 (1953)

thought to be:



It may be that in the presence of ascorbic acid the oxidation of the ring is promoted at the *para* rather than at the *ortho* position with respect to the first hydroxyl They attribute part of the inhibitory action of ascorbate on melanin formation to the reduction of quinones and do not appear to regard its promotion of an alternative pathway of tyrosine metabolism as an important element in this inhibition. Certain melanin pigments can be largely decolorized by ascorbic acid Krueger[441] has shown that ascorbic acid accelerates the initial enzymic oxidation of L-tyrosine and allied substances *in vitro*

Morris, Harpur, and Goldbloom[442] have confirmed earlier observations on deficient infants in particular that pteroylglutamic acid can reduce "tyrosyl" excretion,[443] but Cartwright *et al* [444] could not find a relation between pteroylglutamic acid and ascorbic acid or tyrosine metabolism in deficiency of the former acid in pigs and Gabuzda[444a] found no effect of folinic acid on tyrosyl excretion in a scorbutic patient.

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441 R C Krueger, *J. Am Chem Soc* **72**, 5582 (1950)
442 J E. Morris, E R Harpur, and A Goldbloom, *J. Clin Invest.* **29**, 325 (1950)
443 [illegible]
444 M M Win-
444a [illegible]

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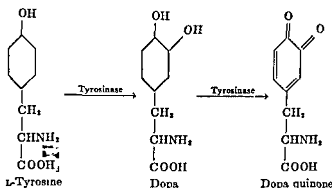
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[448] B. N. La Du and D. M. Greenberg, *J. Biol. Chem.* 190, 245 (1951).
[449] B. Schepartz, *J. Biol. Chem.* 193, 293 (1951).
[450] E. H. Fishberg, *J. Biol. Chem.* 172, 155 (1948).
[451] R. Consden, H. A. W. Forbes, L. E. Glynn, and W. M. Stanier, *Biochem. J.* 50, 274 (1951).
[452] W. E. Knox and M. LeMay-Knox, *Biochem. J.* 49, 686 (1951).
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[450] R Consden, H A W Forbes, L F Glynn, and W M Stanier, *Biochem. J.* 50, 274 (1951)

[451] W E Knox and M LeMay Knox, *Biochem J* 49, 686 (1951)

[451a] B N La Du and D M Greenberg, *Science* 117, 111 (1953).

cationoid properties of oxidized ascorbic acid to other ring systems such as cholesterol and adrenaline, which originates from tyrosine, has been discussed elsewhere It may, however, be worth noting that tyrosine, unlike nine other amino acids examined, produces hypoglycemia in the pigeon,[453] that corticotropin and cortisone raise the excretion of tyrosine in rheumatoid arthritis,[454] and that tyrosine crystals are formed by the addition of a synthetic detergent to the leucocytes but not the erythrocytes of blood[454a] (cf p 386) Collagen has a low tyrosine content.[454b]

Very little is known of the relation, if any, of ascorbic acid to the metabolism of other amino acids and allied substances The reaction between amino acids and ascorbone has been mentioned on p 375. Xanthine oxidase is inhibited by traces of ascorbic acid *in vitro* though little affected *in vivo* [454c] Other miscellaneous relations have been reported.[455, 456, 457]

d. Metabolism of Fatty Acids. In 1947, Bernheim, Wilbur, and Fitzgerald[458] showed that ascorbic acid catalyzed the oxidation, in tissue slices or suspensions, of an unidentified substance, probably fatty,[458a] and that adrenaline inhibited this catalysis Abramson[459] showed that the lowered oxidation of unsaturated fatty acids in the tissues of scorbutic guinea pigs could be corrected by adding ascorbic acid *in vitro* and confirmed the result[458a] that hemoglobin also catalyzed this oxidation. Donnan[460] has obtained similar results in the rat Adrenaline also promotes the oxidation (contrast ref 458) The optimal concentrations of ascorbic acid were fairly low, at about 5 mg per 100 ml These observations on relationships between ascorbic acid and unsaturated fats are of interest in the light of Reid's[461] suggestion that ascorbic acid may be active at cell membranes (e g , in the production of normal collagen) It has some protective action against coloration by oxidation of fat in vitamin E-deficient rats [462] High fat diets

453 G La Grutta, *Boll soc ital biol sper* **27**, 135 (1951)
454 E C Brodie, E B Wallraff, A L Borden, W P Holbrook, C A. L Stephens, Jr , D F Hill, L J Kent, and A R Kemmerer, *Proc Soc Exptl Biol Med* **75**, 285 (1950)
454a W W Ayres, *J Nat Cancer Inst* **10**, 1239 (1950)
454b G R Tristram, *Advances in Protein Chem* **5**, 83 (1949)
454c P Feigelson and B Lines, *J Biol Chem* **201**, 267 (1953)
455 E E Martinson and I V Fetisenko, *Biokhimiya* **4**, 593 (1940)
456 C Fan and T T Woo, *Proc Soc Exptl Biol Med* **45**, 90 (1940)
457 H Christensen and E L Lynch, *J Biol Chem* **172**, 107 (1948)
458 F Bernheim, K M Wilbur, and D B Fitzgerald, *J Gen Physiol* **31**, 195 (1947)
458a F. Bernheim, M L C Bernheim, and K M Wilbur, *J Biol Chem* **174**, 257 (1948)
459 H Abramson, *J Biol Chem* **178**, 179 (1949)
460 S K Donnan, *J Biol Chem* **182**, 415 (1950)
461 M E Reid, *Physiol Revs* **23**, 76 (1943)
462 H Granados, E Aaes-Jørgensen, and H Dam, *Brit J Nutrition* **3**, 320 (1949)

cause increases in ascorbic acid in the interscapular brown adipose tissue of rats, particularly in the cold.[462a] It seems that the relation of ascorbic acid to metabolism of saturated and unsaturated fats will repay further study, though at the moment the experimental facts are difficult to fit into a physiological scheme.

e. Hemopoiesis, Folic Acid, and Vitamin B_{12}; Trace Elements. An association between clinical scurvy and anemia has been observed, though Crandon *et al.*[161] showed if anything a rise in hemoglobin in spite of considerable blood loss during their experiment. Furthermore, intravenous therapy was accompanied by a fall in hemoglobin, which does not seem to be entirely explained by the blood losses entailed for estimations of ascorbic acid in the white layer. It is generally agreed that deficiency of ascorbic acid does not promote erythropoiesis but that deficiency may delay recovery of the hypochromic anemia patient treated with iron alone.

In 1936 Barron and Barron[463] observed that the polycythemia induced in rabbits by feeding cobalt (cf. ref. 464) was diminished by feeding ascorbic acid. This was confirmed by Davis[465] in dogs and by Levey,[466] who caused polycythemia in rats and rabbits by subcutaneously injecting cobalt chloride. In the rat oral or intravenous sodium ascorbate, D-araboascorbate, or D-glucoascorbate had no effect, but in the rabbit intravenous sodium ascorbate markedly, and D-araboascorbate to a small extent, depressed the polycythemia. D-Glucoascorbate was without effect as in the rat. The mechanism of these effects is unknown, but they have gained in interest as the relationships between ascorbic acid, folic acid, and the cobalt-containing vitamin B_{12} have become known. All three vitamins have more or less direct connections with hemopoiesis, the details of which are still being worked out. Only the relationships directly affecting ascorbic acid will be discussed here. The importance of ascorbic acid in nutritional anemia in the guinea pig was shown by Aron.[467] Subsequently, Liu *et al.*[468] found no response in boys with nutritional anemia to ascorbic acid as opposed to iron. Israels[469] studied three scorbutic patients and concluded that they showed a general depression of erythropoiesis and not a failure at any particular stage. Prina and Barbieri[470] claimed that oral administration of

[462a] L. Pagé and L. M. Babineau, *Can. J. Research* 28E, 196 (1950)
[463] A. G. Barron and E. S. G. Barron, *Proc. Soc. Exptl. Biol. Med.* 35, 407 (1936–1937)
[464] K. Waltner and K. Waltner, *Klin. Wochschr.* 8, 313 (1929)
[465] J. L. Davis, *Am. J. Physiol.* 129, 140 (1940)
[466] S. Levey, *Am. J. Physiol.* 158, 315 (1949)
[467] H. C. S. Aron, *J. Nutrition* 18, 375 (1939)
[468] S. H. Liu, H. I. Chu, T. F. Yu, H. C. Hsu, and T. Y. Cheng, *Proc. Soc. Exptl. Biol. Med.* 46, 603 (1941)
[469] M. C. G. Israëls, *Lancet* 244, 171 (1943)
[470] C. Prina and L. Barbieri, *Boll. soc. ital. biol. sper.* 24, 1312 (1948)

$FeSO_4$ and ascorbic acid together to dogs and guinea pigs increases serum iron more than either $FeSO_4$ alone or intravenous ascorbic acid. In 1948 Johnson and Dana[471] published the important result that rats deficient in folic acid gain weight and show a return to normal in white cell count and in normoblasts when ascorbic acid is added to the diet. The reticulocyte count was unaffected and erythrocytes and hemoglobin continued to fall, but the hemorrhagic tendencies noted in nearly every rat were checked by ascorbic acid Subsequent administration of folic acid led to correction of the erythrocyte picture but to no significant increase in the nucleated cells Various interpretations of these results are possible, but it appears that the folic acid-deficient rat is to some extent a scurvy-liable animal. Folic acid and iron requirements are apparently increased in the scorbutic monkey.[471a]

Dietrich *et al* [472] found that the growth of the chick on a semi-purified diet with or without folic acid is stimulated by addition of ascorbic acid plus vitamin B_{12} more than by either separately, and that the folic acid in the liver parallels the stimulation of growth. Folic acid, reciprocally, stimulates the production of vitamin B_{12} in chicks, but the effect of ascorbic acid on this production is equivocal. May, Nelson, and Salmon[473] found that chronic deficiency of ascorbic acid was essential for the production of megaloblastic anemia in monkeys through deficiency of folic acid Ascorbic acid is found to exert an inconsistent growth-promoting effect in the rat-growth assay for vitamin B_{12}[474] and, with other reducing agents, to replace thymidine as a factor in the growth of some lactic acid bacteria[475] (see also p 470); thymidine could also be replaced by vitamin B_{12}

Nichol and Welch[476] made the important observation that, when rat liver is incubated with folic acid, a folic acid derivative is formed, having properties of the *leuconostoc citrovorum* factor (LCF), this formation being augmented by ascorbic and glucoascorbic acids A little later Dietrich, Monson, and Elvehjem[477] showed that oral sulfasuccidine eliminates the growth response of chicks to ascorbic acid—a strong indication that their 1949 effect[472] was due to a microbiological process in the gut The responses

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[473] C D. May, E. N Nelson, and R J Salmon, *J Lab. Clin. Med* 34, 1724 (1949)
[474] D V Tappan, U J Lewis, U D Register, and C. A Elvehjem, *Arch Biochem* 29, 408 (1950).
[475] E Kitay, W S McNutt, and E. E Snell, *J. Bact* 59, 727 (1950).
[476] C. A. Nichol and A. D Welch, *Proc Soc Exptl Biol Med.* 74, 52 (1950)
[477] L S Dietrich, W J Monson, and C A Elvehjem, *Proc. Soc Exptl Biol Med* 75, 130 (1950).

of microorganisms to vitamin B_{12} and ascorbic acid are summarized by Jukes and Stokstad[478] (cf also ref 479).

It now appears that the choline oxidase system is concerned in this very complicated relationship between ascorbic acid, B_{12}, folic acid, and LCF. In 1948 Barrenscheen and von Valyi-Nagy[480] concluded from *in vitro* experiments on muscle that methionine is oxidized to a sulfoxide before transmethylation This is inhibited by cyanide, activated by ascorbic acid, and further activated by heavy metals Later[481] it was shown that the formation of choline from methionine and ethanolamine or serine in liver preparations was activated by ascorbic acid in the presence of cobalt, but not by ascorbic acid alone Barrensheen and Pantlitschko[482] have concluded that oxidation of choline to trimethylamine oxide precedes its action in transmethylation, just as oxidation of methionine is a preliminary

American workers have shown that the choline oxidase system depends on dietary factors Dinning, Keith, and Day[483] showed that the folic acid-deficient monkey has lowered choline oxidase in liver and kidney and suggested that folic acid functions as a prosthetic group in the enzyme system. Handler[484] has shown that in the guinea pig there is no liver choline oxidase and found that fatty liver cannot be established in this animal, and the work of Dubnoff[485] supports the conclusion of Barrenscheen's group that oxidation of choline precedes transmethylation by it Thus transmethylation from choline is probably controlled by choline oxidase Dinning *et al* [486] suggested that Aminopterin displaces folic acid from the bone marrow choline oxidase system Williams[487] has concluded that not folic acid but LCF is the coenzyme of the choline oxidase of rats, though synthetic LCF has less effect on the system *in vitro* than folic acid plus ascorbic acid. None of the effects noted were very large Williams[488] has also shown that rats fed Aminopterin have liver concentrations of ascorbic acid which are less than a half of normal and that low levels of ascorbic acid inhibit and higher levels stimulate choline oxidase activity *in vitro* He concluded (without examining its effects on storage, utilization, excretion, etc) that Aminopterin blocks the endogenous synthesis of ascorbic acid in the rat and that, just as

[478] T H Jukes and E L R Stokstad, *Vitamins and Hormones* 9, 11 (1951)
[479] L S Dietrich, W J Monson, and C A Elvehjem, *Proc Soc Exptl Biol Med.* 77, 93 (1951)
[481] H K Barrenscheen and I Skudrzyk, *Z physiol Chem* 284, 228 (1949).
[482] H K Barrenscheen and M Pantlitschko, *Z physiol Chem* 284, 250 (1949).
[483] J S Dinning, C K. Keith, and P L Day, *Arch Biochem* 24, 463 (1949)
[484] P Handler, *Proc Soc Exptl Biol Med* 70, 70 (1949)
[485] J W Dubnoff, *Arch Biochem* 24, 251 (1949)
[486] J S Dinning, C K Keith, P L. Davis, and P L Day, *Arch Biochem.* 27, 89 (1950)
[487] J N Williams, Jr , *J Biol Chem* 191, 123 (1951)
[488] J N Williams, Jr , *Proc Soc Exptl Biol Med.* 77(2), 315 (1951).

$FeSO_4$ and ascorbic acid together to dogs and guinea pigs increases serum iron more than either $FeSO_4$ alone or intravenous ascorbic acid. In 1948 Johnson and Dana[471] published the important result that rats deficient in folic acid gain weight and show a return to normal in white cell count and in normoblasts when ascorbic acid is added to the diet. The reticulocyte count was unaffected and erythrocytes and hemoglobin continued to fall, but the hemorrhagic tendencies noted in nearly every rat were checked by ascorbic acid Subsequent administration of folic acid led to correction of the erythrocyte picture but to no significant increase in the nucleated cells. Various interpretations of these results are possible, but it appears that the folic acid-deficient rat is to some extent a scurvy-liable animal. Folic acid and iron requirements are apparently increased in the scorbutic monkey[471a]

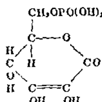
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cortical activity, seem to assume that a scorbutic animal is devoid of ascorbic acid. There is no evidence for this, or that any mammal can survive for a moment in total absence of an ascorbic acid.

f. Oxidation of Gluconic Acid. *In their scheme of oxidation of* D-glucose-6-phosphate in animal tissues Dickens and Glock[497] include as a hypothetical intermediate the ene-diol with the structure.



this is isomeric with the 6-phosphate of D-araboascorbic acid, which is a 1-lactone Horecker, Smyrniotis, and Seegmiller[498] posit a hypothetical 3-keto acid in the oxidation of 6-phosphogluconate by yeast 3-Keto acids very rapidly change into the isomeric ascorbic acid[30] Scott and Cohen[499] have pointed to the existence of an ascorbic-acid-like substance in this oxidation by yeast

These suggestions are interesting because D-araboascorbate has a twentieth of the antiscorbutic activity of the true vitamin, L-xyloascorbate, and because there is some similarity in the processes leading to increased production of ascorbic acid and glucuronic acid in the rat (see p 444) The chloretone-treated rat produces true vitamin C, not D-araboascorbate, however, and the scorbutic guinea pig can synthesize glucuronic acid[500] Glucuronic acid is formed from glucose by oxidation at the 6 atom, gluconic by oxidation at the 1 atom, these processes are quite distinct, but the relationships between the hexose acids are relevant to the mechanism of the production of ascorbic acid from glucose *in vivo* (see p 445)

g. Peroxidases and Catalases. Marks[501] showed that ascorbic acid hastens the inactivation of catalase, and in 1943 Lemberg and Legge[502] showed that ascorbic acid inhibited crystalline horse liver catalase by accelerating its destruction by hydrogen peroxide Of the substrates of Agner's[503] verdoperoxidase, an iron-containing green protein isolated from leucocytes, ascorbic acid was the only animal metabolite of importance, the others

[497] F Dickens and G E Glock, *Biochem J* **50**, 81 (1951)
[498] B L Horecker, P Z Smyrniotis, and J E Seegmiller, *J Biol Chem* **193**, 383 (1951)
[499] D B Scott and S S Cohen, *J Biol Chem* **188**, 509 (1951)
[500] A J Quick, *J Biol Chem* **100**, 411 (1933)
[501] G W Marks, *J Biol Chem* **115**, 299 (1936)
[502] R Lemberg and J W Legge, *Biochem J* **37**, 117 (1943)
[503] K Agner, *Advances in Enzymol* **3**, 137 (1943)

ascorbic acid is involved in the synthesis of LCF (see ref. 476), LCF or folic acid is involved in the synthesis of ascorbic acid More recently Schwartz *and Williams*[489] have shown that, once Aminopterin has caused signs of deficiency, LCF and folic acid have little effect on the low levels of ascorbic acid in rat liver or on reversal of the deficiency. The effect of ascorbic acid was not examined

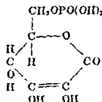
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Ascorbic acid has been shown by Jurgens and Studer[494] to stimulate leucopoiesis in rats with a folic acid deficiency induced by succinyl-sulfathiazole (cf ref 471), and Tanaka[495] has shown that ascorbic acid can liberate iron from ferritin *in vitro*, and that in guinea pig liver brei the enzymic process concerned in the liberation of iron is inhibited by folic acid, sodium fluoride, and iodoacetic acid. The relation of ferritin to thiol oxidation in the liver has been examined by Mazur, Litt, and Shorr.[496] Certain aspects of the relationships of vitamin B_{12} and ascorbic acid in bacteria are discussed on p 470 In a recent paper[496a] it has been shown that oral ascorbic acid increases the conversion of folic acid to folinic acid, as revealed by urinary excretion of the latter, in man. Hill and Scott[496b] consider that ascorbate's primary role in this context in chick liver homogenates is in liberating combined LCF The conversion of folic to folinic acid is greatly reduced in human scurvy[496c] Other workers[496d] have concluded from what *appears to be inadequate evidence that ascorbate is not essential* in the monkey for the conversion of folic to folinic acid. As a cautionary note it may be remarked that some workers in this field, and in the field of adrenal

489 M A Schwartz and J N Williams, Jr , *J Biol Chem* 194, 711 (1952)
491 D S Gaines, H P Broquist, and W L Williams, *Proc Soc Expll Biol Med.* 77, 247 (1951)
492 G R Drysdale, J J Betheil, H A Lardy, and C A Baumann, *Arch Biochem Biophys* 33, 1 (1951)
494 [illegible]
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496
496a [illegible] *Med* 38, 95 (1951)
496b C H Hill and M L Scott, *J Biol Chem* 196, 195 (1952)
496c G. J Gabuzda, Jr , G B Phillips, R F Schilling, and C S Davidson, *J Clin Invest* 31, 756 (1952)
496d C D May, A Hamilton, and C T. Stewart, *J. Lab Clin Med* 40, 924 (1952).

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[502] G W Marks, *J Biol Chem* 115, 299 (1936)
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[491] D S Gaines, H P Broquist, and W L Williams, *Proc Soc Exptl Biol Med* **77**, 247 (1951)
[492] G R Drysdale, J. J Betheil, H A Lardy, and C A Baumann, *Arch Biochem Biophys* **33**, 1 (1951)
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[496] [illegible] (1950)
[496a] [illegible] *Lab Clin Med* **38**, 95 (1951).
[496b] C H Hill and M L Scott, *J Biol Chem* **196**, 195 (1952)
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ticularly in his studies on the effects of substances chemically related to ascorbic acid (see p 379) Day[520] found that alkaline phosphatase and ascorbic acid were rarely to be found together in the cells of insects, and, if together, they were in different positions in the cell A diet containing 10% D-glucoascorbic acid had little effect on the phosphatase of *Blattella.*

Ram and Giri[521] claim that ascorbic acid, especially in the presence of copper, inhibits phosphorylase

k. Muscle and General Cellular Metabolism. Fatigue has long been recognized as a characteristic symptom of scurvy in man,[104] and a few observations have been made on the relation of ascorbic acid to muscular contraction Basu and Biswas[523] claimed that ascorbic acid improves muscular function, and Friedman[524] showed that smooth muscle in scurvy shows a greatly reduced reaction to histamine The diminished effects of adrenaline in scurvy have already been discussed (p 417) Minz and Passouant[525] claim that ascorbic acid, with quinine, improves myotonia, and Lagos[526] states that ascorbic acid, among a group of vitamins, inhibits contractions in the isolated guinea pig gall bladder The inactivation of actin is inhibited by ascorbic acid and other reducing agents.[527]

Few of the enzyme systems concerned in the provision of energy have been related to ascorbic acid The scorbutic guinea pig has a decreased succinic dehydrogenase activity in heart and skeletal muscle [528] Glycolysis by mouse brain homogenates is inhibited by a factor requiring iron and either cysteine or ascorbic acid, this factor apparently destroying glyceraldehyde phosphate dehydrogenase [529] It has also been shown that ascorbic acid or cysteine is needed for the stabilization and enhancement of aconitase activity by iron [530] Judah[530a] has recently made the important observation that in mitochondrial systems from rat liver the oxidation of reduced cytochrome *c* in the presence of ascorbate, which is very probably oxidized to ascorbone, is accompanied by phosphorylation (see also p. 417.)

l. Clotting. In his treatise Lind[531] stated that the blood of scorbutics was unclotted after death, but there are no recent reports of increased clotting or bleeding times in scorbutic men or guinea pigs Many experimental

[520] M F Day, *Australian J Sci Research* 2B, 31 (1949)
[521] J S Ram and K V Giri, *Current Sci (India)* 18, 440 (1949)
[523] N M Basu and P Biswas, *Indian J Med Research* 28, 405 (1940)
[524] H J Friedman, *J Allergy* 12, 221 (1941)
[525] B Minz and P Passouant, *Rev can biol* 4, 510 (1945)
[526] D A Lagos, *Rev españ fisiol* 5, 47 (1949)
[527] F B Straub and G Feuer, *Biochim et Biophys Acta* 4, 455 (1950).
[528] C J Harrer and C G King, *J Biol Chem* 138, 111 (1941)
[529] I Krimsky and E Racker, *J Biol Chem* 179, 903 (1949)
[530] S R Dickman and A A Cloutier, *J Biol Chem* 189, 379 (1951)
[530a] J D Judah, *Biochem J* 49, 271 (1951)
[531] J Lind, A Treatise of the Scurvy, Edinburgh, 1753

mentioned being catechol, pyrogallol, hydroquinone, and *p*-phenylenediamine

Chance[505, 506] has shown that in the oxidation of ascorbic acid by horseradish peroxidase there are two independent reactions which may be simultaneous First, a peroxidase-peroxide complex can react directly with ascorbic acid, and, second, a phenol can act as a cyclically oxidized and reduced intermediary Dilute ascorbic acid accelerates the breakdown of a catalase-hydrogen-peroxide complex by forming a secondary complex and not by *direct acceptance of a hydroxyl radical.*

h. Cholinesterase. Von Pantschenko-Jurewicz and Kraut[507] showed that ascorbic acid had in some respects the property of a coenzyme for liver esterase, and they suggested that it was a precursor of the coenzyme. There have been claims that ascorbic acid restores serum cholinesterase activity[508] and that hypervitaminosis C tends to increase serum cholinesterase [509]

i. Histamine. Very dilute ascorbic acid had no effect as an antihistamine on the isolated guinea-pig intestine,[510] though García[511] showed a potentiating effect on histamine under certain conditions, and ascorbic acid failed to affect aerosol-histamine shock in the guinea pig [512] Some flavonoid compounds potentiate the inhibition of histidine decarboxylase by ascorbic acid *in vitro*.[513] Businco[514] concludes that ascorbic acid and nicotinamide are the most effective antihistaminic substances in guinea pigs *Histamine is known to depress the ascorbic acid content of the adrenal* cortex (see p 411 and p 443)

j. Phosphatase and Phosphorylase. Phosphatase activity is lowered in the serum of scorbutic[515] and in healing skin in C-deficient[516] guinea pigs. Perkins and Zilva[518] have concluded that ascorbic acid does not *per se* regulate the phosphatase content of tissues in the guinea pig Gould[519] has used the serum phosphatase as a sensitive indicator of early scurvy, par-

505 B Chance, *Arch Biochem* **24**, 389 (1949).
506 B Chance, *J Biol Chem* **182**, 649 (1950).
507 W von Pantschenko-Jurewicz and H Kraut, *Biochem Z* **285**, 407 (1936)
508 E Frommel and J Piquet, *Acta Pharmacol Toxicol* **3**, 31 (1947)
[illegible]
513 G. J. Martin, M Graff, R Brendel, and J M Beiler, *Arch Biochem* **21**, 177 (1949)
514 L Businco, *Boll soc ital biol. sper.* **25**, 274 (1949)
515 E N Todhunter and W. Brewer, *Am J. Physiol.* **130**, 310 (1940).
516 J. F. Danielli, H B Fell, and E Kodicek, *Brit J Exptl Path.* **26**, 367 (1946).
518 H R. Perkins and S S. Zilva, *Biochem J* **47**, 306 (1950)
519 B S Gould, *Arch Biochem* **19**, 1 (1948).

that the tissues of chloretone-treated rats could synthesize ascorbic acid *in vitro*

Sutton, Kaeser, and Hansard[546] found that chloretone-treated rats lacking ovaries (corpus luteum), pituitary, and adrenals still synthesized ascorbic acid and thus confirmed the statement[547] that the adrenal is not the sole site of synthesis of ascorbic acid Probst and Schultze[548] have shown conclusively by isolation from urine of pure L-ascorbic acid in 10% yield and of certain derivatives in high yield that chloretone stimulates the production of true vitamin C by the rat and not, for example, of D-araboascorbic acid (cf. p 441) Jackel *et al* [549] have shown that approximately 0.3% of C^{14}-labeled glucose administered to the rat becomes ascorbic acid in 24 hr and that chloretone makes no contribution to the molecule The whole glucose chain, without breaking, was thought to be used in the synthesis of ascorbic acid, though a recent paper[550] suggests that the C_1 atom in ascorbic acid does not come solely from the C_6 or C_1 atom of glucose Very recently King's group[550a] has shown that D-glucose is almost certainly a direct precursor of ascorbic acid, the 6-carbon of which is derived from the 1-carbon of glucose

n. The Fate of Ascorbic Acid. It is commonly and probably erroneously thought, from work on the pure chemistry of ascorbic acid and ascorbone,[18, 271] that *in vivo* ascorbic acid is mainly destroyed by the irreversible hydrolysis of the lactone ring of ascorbone to diketogulonic acid, followed by destruction of the latter compound Damron, Monier, and Roe[76] have examined the amounts of ascorbate, ascorbone, and diketogulonate present in the guinea pig after intraperitoneal injection and conclude that the reactions


Ascorbate ⇄ Ascorbone → Diketogulonate

do not constitute the main pathway of the disposal of ascorbate, but their results are equally consistent with the conclusion that only the second reaction, giving diketogulonate, is not a part of this pathway.

Their failure to refer to the work of those who have suggested that oxalate is a product of the metabolism of ascorbate is a curious omission. Flaschenträger and Müller[551] were the first to suggest that ascorbate gave

[545] C V Smythe and C G King, *J Biol Chem* 142, 529 (1942)
[546] T S Sutton, H E Kaeser, and S L Hansard, *J. Biol Chem* 144, 183 (1942).
[547] H M Vars and J J Pfiffner, *Proc Soc. Exptl Biol Med* 31, 830 (1934)
[548] G W Probst and M O Schultze, *J. Biol. Chem* 187, 453 (1950)
[549] S S Jackel, E H Mosbach, J J Burns, and C G King, *J Biol Chem* 186, 569, (1950)
[550] C G King, H. B Burch, H Horowitz, and J Douglas, *Science* 114, 492 (1951).
[550a] H H Horowitz, A P Doerschuk, and C. G King, *J Biol Chem* 199, 193 (1952)
[551] B Flaschenträger and P B Müller, *Z physiol Chem* 251, 52 (1938).

incisions have been made in both species without prolonged hemorrhage. It has been stated that plasma fibrinogen rises in scurvy[532] and that ascorbic acid has a promoting effect on thrombin.[533]

m. The Origin of Ascorbic Acid. The reactions leading to the production of ascorbic acid in the scurvy-insensitive species and in plants remain unknown. The classical methods of laboratory synthesis throw little light on the problem: in them the most widely used starting material is glucose, a D-hexose whose carbon chain is in effect inverted to give ascorbic acid, which is classed with the L-hexoses. It is perhaps possible that ascorbic acid is made via ascorbone and that this is formed by reversal of the phosphate-catalyzed reaction demonstrated by Rosenfeld.[534] The method of synthesis of Helferich and Peters[35] bears a rather distant parallelism to this hypothetical reversal, but the bulk of the experimental work favors a 6-carbon precursor of ascorbic acid. Early work was reviewed by King.[535]

In 1935 Guha and Gosh[536] claimed that mannose was a precursor of ascorbic acid in its formation by rat tissues, but Hawthorne and Harrison[537] and others[538] could not confirm this. Rudra (e.g., ref. 539) in 1939 claimed that manganese promoted the *in vitro* production of ascorbic acid by animal preparations, but Boyer, Shaw, and Phillips[540] could not confirm this. Ruffo and Tartaglione[541] showed that *in vitro* glucose, mannose, fructose, gluconate, and 2-ketogluconate, but not D-galactose or L-sorbose, gave ascorbic acid in the presence of minced rat kidney and liver, and Babbar[542] has concluded that glucose is the probable precursor of ascorbic acid in *in vitro* experiments of this type.

An important observation on the production of ascorbate in the whole animal was made in 1939 by Musulin *et al.*[543] when they discovered that certain unsaponifiable extracts caused increased excretion of ascorbic acid in rats. By giving 20 mg. of chloretone (CCl_3—$COH(CH_3)_2$) per day, excretion could be raised from 0.2 to 50 or 60 mg.[544] Smythe and King[545] showed

[532] W. R. Sullivan, E. O. Gangstad, and K. P. Link, *J. Biol. Chem.* **152**, 367 (1944).
[533] G. Weber and K. Drechsler, *Am. J. Physiol.* **162**, 665 (1950).
[534] B. Rosenfeld, *J. Biol. Chem.* **150**, 281 (1943).
[535] C. G. King, *Cold Spring Harbor Symposia Quant. Biol.* **7**, 137 (1939).
[536] B. C. Guha and A. R. Gosh, *Nature* **135**, 234, 871 (1935).
[537] J. R. Hawthorne and D. C. Harrison, *Biochem. J.* **31**, 1061 (1937).
[538] [illegible] *Biochem. Z.* **291**, 209 (1937).
[539] [illegible]
[540] [illegible] *Chem.* **143**, 417 (1942).
[541] A. [illegible] 283 (1948) [*C. A.* **43**, 6265 (1949)].
[542] I. J. Babbar, *Indian Med. Gaz.* **84**, 540 (1949).
[543] R. R. Musulin, R. H. Tully, 3rd, H. E. Longenecker, and C. G. King, *J. Biol. Chem.* **129**, 437 (1939).
[544] H. E. Longenecker, H. H. Fricke, and C. G. King, *J. Biol. Chem.* **135**, 497 (1940).

thermore, scurvy will not cause abortion in the guinea pig if instituted after the early part of pregnancy (p 419), a result probably explained by the dispensability of the corpus luteum at later stages Cord blood usually contains more ascorbate than does the mother's blood. Whether or not intrauterine ascorbic acid is synthesized, there is no reason for supposing that its presence is not essential for the production of fetal connective tissue (cf refs 2, 352, 557, 558)

There have been a few observations on the effect of ascorbic acid on cell proliferation Shapiro[561] reported that vitamin C, in physiological amounts, retarded cell division in sea urchin eggs, and Raunich[562] has shown that the tails of various tadpoles regenerate more rapidly in a dilute solution of ascorbic acid than in distilled water. Ogino[563] has shown that, in the incubated egg, glucose promotes synthesis of ascorbic acid, this promotion being increased if manganese dichloride is given simultaneously

p. Tumors and Leukemia. The discussion of ascorbic acid and neoplasms falls naturally into two parts—the effect of ascorbic acid on the course of neoplasms, and the effect of neoplasms on ascorbic acid

Since the early days of synthetic ascorbic acid some tumor tissues have [illegible]

as[illegible]

of [illegible]

gested that ascorbate was responsible Not all tumors are high in ascorbate [568]

Elson, Kennaway, and Tipler[569] showed that treating rats with 1,2-5,6-dibenzanthracene causes an increase in liver ascorbic acid which is possibly related to a profound change in basal metabolism Dyer and Ross[570] found that ascorbone and diketogulonate were low in extracts of a variety of transplanted tumors in the mouse In nearly all tumors ascorbate per gram of nitrogen and the ascorbone-ascorbate ratio were higher than in the corresponding livers Lymphosarcoma implantation did not consistently affect the adrenal ascorbate of male mice [571] Cater[572] has found a decrease

[557] A St G Huggett, *Physiol Revs* 21, 438 (1941)
[558] M T Harman and L E Warren, *Trans Kansas Acad Sci* 54, 42 (1951)
[561] H Shapiro, *Biol Bull* 94, 79 (1948)
[562] L Raunich, *Ricerca sci* 20, 1502 (1950)
[563] S Ogino, *Kagaku* 21, 592 (1951)
[564] E Boyland, *Biochem J* 27, 802 (1933)
[565] S. Edlbacher and A Jung, *Z physiol Chem* 227, 114 (1934)
[566] C Frisch and R Willheim, *Biochem Z* 277, 149 (1935)
[567] J P Greenstein, *Advances in Enzymol* 3, 315 (1943)
[568] L A Elson, E L Kennaway, and M M Tipler, *Brit J Cancer* 3, 148 (1949).
[570] H M Dyer and H E Ross, *J Natl Cancer Inst* 11, 313 (1950).
[571] E Adams and A White, *Proc Soc Exptl Biol Med* 76, 322 (1951).
[572] D B Cater, *J. Path Bact* 63, 209 (1951).

rise to oxalate in urine, and Scheinkman[552] found that the urinary excretion of oxalate rose after the administration of ascorbic acid to guinea pigs Rosenfeld showed in a very important paper in 1943[534] that, *in vitro* at pH 7 0 in the presence of phosphate, ascorbone disappeared not by hydrolysis to diketogulonate but stoichiometrically by direct fission of the molecule to oxalate and a C_4 compound In the presence of oxygen an oxalyl compound, possibly oxalylthreonate, is formed first. In the absence of phosphate the conversion of ascorbone to another C_6 compound was confirmed Cyanide could replace phosphate in the production of oxalate Rosenfeld[534] pointed out that the 27 mg per day of oxalic acid found by Dodds and Gallimore[553] in human urine corresponded with the destruction of 50 mg of ascorbate In a contemporary paper Barrett[554] showed that the addition of ascorbic acid to human blood *in vitro* resulted in an increase in the oxalate content The half-life of ascorbone under physiological conditions is in some doubt Kinsey[77b] found it to be about 20 min at pH 7 3 in phosphate buffer at 37°, *and 50 min in beef aqueous humor, values at least three times those* of Penney and Zilva[555] and of Ball.[39]

The proof that urinary oxalic acid is at least partly derived from dietary ascorbic acid was given by Burns, Burch, and King,[153] who fed ascorbic acid labeled with C^{14} in the 1-C atom to normal and scorbutic guinea pigs Twenty to thirty per cent of the label appeared in the respiratory carbon dioxide, and 3 to 6% in the urine, of the latter 60% was in the form of oxalate Glucose can also yield oxalic acid as an end product A guinea pig excreted about 8% of its daily intake of ascorbic acid as oxalate. The final establishment of oxalate as one end product of the metabolism of ascorbic acid opens up new lines of work, though rapid movement along them depends on the development of methods for oxalate more convenient than those of Dodds and Gallimore [553] A measurement of the oxalic acid in the adrenal effluent blood, particularly after administration of corticotropin, *would be interesting, and the relation of oxalate production to the metab*olism of calcium may be important.

L-Ascorbic acid is not a precursor of glucuronic acid [556]

o. **Fetus and Growth.** Nutritional deficiencies almost invariably limit

[illegible]

the maturation of the fetus has been in some doubt, as it has been thought possible that ascorbic acid is synthesized inside the pregnant uterus Fur-

552 [illegible] (1949)
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been generally confirmed Javillier and Lavollay[584] suggested that vitamin P delays the oxidation of adrenaline *in vivo* and thus affects capillary blood flow; later workers have not agreed about this action

Beiler and Martin[585] have found that rutin inhibits hyaluronidase *in vitro*, and that this and other P compounds promote the inhibitory effect of ascorbic acid Elster, however,[586] failed to find inhibition of hyaluronidase by rutin in the rat The sparing action of vitamin P on vitamin C claimed in several papers was found by Crampton and Lloyd[587] only when sub-optimal amounts of vitamin C were fed, but Clark and Geissman[588] found that rutin and C together had an activity three or four times that of either one alone in potentiating the action of adrenaline on the recovery time of rabbit ileum Papageorge and Mitchell[589] have found that rutin given orally to guinea pigs receiving adequate ascorbic acid increased the ascorbate in the adrenals but not in other organs (see also ref 590)

In a nutritional study Zilva[591] concluded that no vitamin P exists The effect of maclurin in causing a transient rise in capillary resistance after injection is apparently pharmacological In support of this, Bohr, McIvor, and Rinehart[592] state that hesperidin causes a fall in blood pressure due to visceral dilatation, accompanied by vasoconstriction in the skin vessels.

The conclusion that vitamin P affects the action of adrenaline has in the main been confirmed by recent work (cf p 391)[593, 594]

In summary it may be concluded that in a scorbutic animal "P" may increase the sensitivity of capillaries or precapillaries to adrenaline, which in the absence of ascorbic acid appears to be less active as a vasoconstrictor or in promoting vasomotion

r. Vitamin R. Rygh and Rygh in 1932[595] claimed that methylnornarcotine had antiscorbutic properties, but in 1933 Dunn[596] refuted this In 1949 Rygh[597] claimed that methylnornarcotine does not lengthen life in the absence of ascorbic acid but that it excludes the presence of the signs of

[584] M Javillier and J Lavollay *Helv Chim Acta* **29**, 1283 (1946)
[585] J M Beiler and G J Martin, *J Biol Chem* **174**, 31 (1948)
[586] S K Elster, *Proc Soc Exptl Biol Med* **71**, 15 (1949)
[587] E W Crampton and L E Lloyd, *Federation Proc* **9**, 355 (1950)
[588] W G Clark and T A Geissman, *J Pharmacol Exptl Therap* **95**, 363 (1949)
[589] E Papageorge and G L Mitchell, Jr, *J Nutrition* **37**, 531 (1949)
[590] A M Ambrose and F De Eds, *J Nutrition* **38**, 305 (1949)
[591] S S Zilva, *Biochem J* **45**, 79 (1949)
[592] D F Bohr B C McIvor, and J F Rinehart, *J Pharmacol* **97**, 243 (1949).
[593] J M Crismon, R B Berry J D Mcbien, and F A Fuhrman, *Am J Physiol* **164**, 391 (1951)
[594] A A Schiller, *Am J Physiol* **165**, 293 (1951)
[595] O Rygh and A Rygh, *Z physiol Chem* **204**, 114 (1932)
[596] W J Dunn *Biochem J* **27**, 233 (1933)
[597] O Rygh, *Bull soc chim biol* **31**, 1403 (1949).

of ascorbate in the preen gland, no change in the adrenals, and an increase in the ovaries of young hens bearing Rous's sarcoma. Mettier, Ellenhorn, and Gordan[573] found no effect in four patients with chronic lymphatic leukemia after injections of desoxycorticosterone and ascorbic acid The high concentrations of ascorbic acid in some leukemic white cells have already been discussed (pp. 386 and 387)

Deficiency of ascorbate has been shown to result in necrotic changes and slower growth in transplantable fibrosarcoma in guinea pigs.[575] Muller-Lenhartz[576] recommends auxiliary treatment of carcinoma with injected ascorbic acid and vitamin A, general condition and tolerance to X-rays are improved Selle[577] has examined the effect of ascorbic acid on resistance to radiation Ascorbic acid increased reduced glutathione in leucocytes but not in erythrocytes in malignant melanoma [578] Some interesting work has been done by Warren[295] on the autoxidation of 3,4-benzopyrene *in vitro* in aqueous acetone solution This is extremely slow, but it is greatly hastened by the addition of ascorbic acid

q. Vitamin P. Scarborough and Bacharach reviewed "vitamin P" in 1949[579] and concluded that there was some evidence that vitamin P could *affect capillary strength but that it was not necessarily a vitamin; it cannot* modify the course of scurvy in the guinea pig. In their original report Armentano *et al* [580] concluded that the activity of vitamin P in promoting recovery in a variety of capillary abnormalities was distinct from that of ascorbic acid Scarborough[581] found similar results in man, and Selezevena[582] made the important observation that, in the perfused leg of the guinea pig, dietary lemon juice, but not ascorbic acid, appeared to cause intermittency of outflow of the perfusion fluid and hence, apparently, a reduction in edematous accumulation Bourne[583] showed that the capillary strength of guinea pigs on scorbutigenic diets supplemented with ascorbic acid decreased below normal and was increased by citrin, and these results have

[573] S R Mettier, M J Ellenhorn, and G Gordan, *Blood* 5, 1156 (1950).

[575] W van B Robertson, A J Dalton, and W. E Heston, *J Natl Cancer Inst* 10, 53 (1949)

[576] W Muller Lenhartz, *Z ges inn Med* 4, 267 (1949) [*Brit Abstr AIII* 1950, 735]

[577] W A Selle, *U S Atomic Energy Comm Rept* NEPA-1127 (1949), *Nuclear Sci Abstracts* 4, 217 (1950)

[578] A Tzanck, M Steinbuch, and G Melki, *Bull soc franç dermatol syphilig* 58, 291 (1951)

[579] H Scarborough and A L Bacharach, *Vitamins and Hormones* 1, 1 (1949)

[580] L Armentano, A Bentsath, T Béres, St Rusznyák, and A Szent-Györgyi, *Deut med Wochschr* 62, 1325 (1936)

[581] [illegible] *Lancet* 238, 644 (1940)

[582] [illegible] *earch U S S R* 3, 198, 205 (1941)

[583] [illegible]

secondary antitoxin response to diphtheria toxoid Earlier reports that complement depends on ascorbic acid have been discredited by Kodicek and Traub[615] and by Rice and Boulanger [616] The relation of ascorbate and phagocytosis has been discussed on p 388; Spink *et al* [617] have shown that the bactericidal properties of human blood *in vitro* are unrelated to its concentration of ascorbate Ascorbate is bactericidal in rather high concentrations to virulent strains of *M tuberculosis*.[618] In lower concentrations ascorbic acid retards the growth of human but not bovine tuberculosis bacteria [619]

It seems likely that any effect ascorbic acid may have in infections is related to its role in adrenal cortical function, phagocytosis, and the reaction of tissues to injury The scorbutic animal is certainly more liable to infection than the non-scorbutic, the value of vitamin C in doses above these preventing clinical scurvy remains conjectural in relation to infection

In 1948 a Medical Research Council committee[128] reported that acne may be exacerbated in the later stages of a C-deficient diet at the onset of clinical scurvy

t. General Metabolism. The function of ascorbic acid as a respiratory catalyst is still problematical· indeed it seems likely that its role does not lie in the main pathways by which oxygen is reduced in energy-yielding processes, but in special side-lines of oxidation such as that of tyrosine Nevertheless there is, in the movement of ascorbone into erythrocytes, the central nervous system, and the anterior chamber of the eye, a suggestion that ascorbone may behave as an important oxygen-carrier It is tempting to speculate that its entry into erythrocytes provides in oxygen lack a possible mechanism for oxygen secretion from the lung, a mechanism that may be related to the observations of Bronstein (p. 395) and of Boutwell *et al* (p 399)

Streck[622] claimed that ascorbic acid increased the respiratory quotient and the oxygen uptake of dogs Large doses caused shivering The oxygen uptake of men also increased, though less markedly and without a change in respiratory quotient Osborne and Farmer[623] found that pyrexia in fever cabinets did not lead to a significant change in plasma ascorbate, and Weaver's[624] claim that 100 mg ascorbic acid per day can prevent heat prostration apparently has not been confirmed Wachholder, in an impor-

[615] E Kodicek and B Traub, *Biochem J* 37, 456 (1943)
[616] C E Rice and P Boulanger, *Can J. Research* 28E, 262 (1950)
[617] W W Spink, S Agnew, O Mickelsen, and L Dhal, *J Immunol* 44, 303 (1942).
[618] P Pichat and A Reveilleau, *Compt rend soc biol* 144, 1386 (1950).
[619] N M Sokolova and M A Linnikova, *Zhur Mikrobiol Epidemiol Immunobiol* 9, 76 (1947) [C A 43, 7101 (1949)].
[622] F Streck, *Biochem Z* 277, 279 (1935)
[623] S L Osborne and C J Farmer, *Proc Soc Exptl Biol Med* 49, 575 (1942).
[624] W L Weaver, *Southern Med. J.* 41, 479 (1948).

scurvy at death. In 1951[597] he claimed that "vitamin R" (methylnornarcotine) slightly retards the growth of cancer in mice and that it "neutralizes" the toxic effects of certain amino acids in scurvy.[597a] The evidence for the antiscorbutic properties of vitamin R remains vanishingly thin and may be compared with that for other substitutes (see p. 378).

s. Infection. The work of Long and his collaborators on the sensitivity of the BCG-treated guinea pig to tuberculin (see p. 417) provides some direct evidence of a possible role of ascorbic acid in the relationships of the animal to invading organisms. Since the work of Harris and his collaborators,[598, 599, 600] it has been known that the dose of ascorbic acid needed for saturation is increased in a variety of infections, but the evidence that supplements of vitamin C increase "resistance" to infection is slight Scheunert[601] found that a daily supplement of 100 mg, but not of 50 mg, ascorbic acid led to slight improvements in health of factory workers. It would be well worth while to conduct further large-scale experiments of this type

Vitamin C has been used as an adjuvant to gold therapy asthma,[602] and Abbasy *et al* [598] showed a decreased excretion in osteomyelitis Tuberculosis has received considerable attention, apart from the work of Long already mentioned Abbasy, Harris, and Ellman[599] demonstrated a decreased excretion in pulmonary tuberculosis Babbar[605] found that 200 mg. ascorbic acid per day given orally led to more frequent gains in weight and increases in erythrocyte count, although sedimentation rates were unaffected The effect of vitamin C has been examined in diphtheria[606, 608, 609] and in whooping cough [610] Vitamin C relieves pain in zoster[611] and apparently improves chronic aphthosis [612]

Bourne[613] considers that ascorbate may be of immunological significance, and Long[614] has shown that C-deficient guinea pigs show a greatly reduced

597 [illegible] (1951)
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598 [illegible] 77 (1937)
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608 [illegible] 30, 1068
(1940)
[illegible] 417 (1940)

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[617] W. W. Spink, S. Agnew, O. Mickelsen, and L. Dhal, *J. Immunol.* 44, 303 (1942).
[618] P. Pichat and A. Reveilleau, *Compt. rend. soc. biol.* 144, 1346 (1950).
[619] N. M. Sokolova and M. A. Lasnikova, *Zhur. Mikrobiol. Epidemiol. Immunobiol.* 9, 76 (1947) [*C. A.* 43, 7101 (1949)].
[621] F. Streck, *Biochem. Z.* 277, 279 (1935).
[622] S. L. Osborne and C. J. Farmer, *Proc. Soc. Exptl. Biol. Med.* 49, 575 (1942).
[623] W. L. Weaver, *Southern Med. J.* 41, 479 (1948).

tant paper,[624a] has shown that exercise, in several species, leads to an increased consumption of vitamin C and a redistribution of ascorbate and ascorbone throughout the body.

On an adequate diet men exposed to cold showed only slight changes in metabolism of ascorbate,[625] but Dugal and his collaborators have shown that exposure to cold increases excretion of ascorbate in rats but decreases it in guinea pigs[626] and that large doses of ascorbic acid accelerate thyroid enlargement and the diminution of adrenal cholesterol, increase spleen weight, partly inhibit thymic atrophy, and lessen hypotension and adrenal hypertrophy [627] Rats in the cold increase their excretion of ascorbate by 53%, of ascorbone by 186%, and of diketogulonate by 500% [627a] Large doses of ascorbic acid assist acclimatization of monkeys to cold.[627b] The excretion of ascorbone and diketogulonate by rats is greatly increased after exposure to X-rays.[627c]

V. Metabolism in Plants

1. Ascorbic Acid Oxidase

Tauber, Kleiner, and Mishkind[628] reported an enzyme from Hubbard squash that specifically catalyzed the reaction between oxygen and ascorbate, and in 1940 Lovett-Janison and Nelson[629] prepared a purified oxidase as a copper-protein from summer squash Tadokoro and Takasugi claimed in 1941[630] that the enzyme was probably a nucleoprotein, but this is not accepted by Dunn and Dawson [631] Powers, Lewis, and Dawson[632] in 1944 found that the enzyme is blue and some thousand times as active as its equivalent of inorganic copper Its activity decreases as the oxidation of the substrate proceeds. In 1945 the existence of the enzyme was questioned,[633] [634] but since this stimulating work on model systems a series of papers from a variety of laboratories have made it clear that an enzyme

[624a] K Wachholder, *Arbeitsphysiologie* 14, 342 (1951)
[625] R M Kark, R R M Croome, J Cawthorpe, D M Bell, A Bryans, R J MacBeth, R E Johnson, F C Consolazio, J L Poulin, F. H L Taylor, and R C Cogswell, *J Applied Physiol* 1, 73 (1948)
[626] M Thérien and L P Dugal, *Rev can biol* 8, 248 (1949)
[627] M Thérien, J Leblanc, O Héroux, and L P Dugal, *Can. J Research* 27E, 349 (1949)
[627a] M M Monier and R J Weiss, *Proc Soc Exptl Biol Med* 80, 446 (1952)
[627b] L -P Dugal and G Fortier, *J. Applied Physiol* 5, 143 (1952)
[627c] M M Monier and R J Weiss, *Proc Soc Exptl Biol Med* 81, 598 (1953)
[628] H Tauber, I S Kleiner, D A Mishkind, *J Biol Chem* 110, 211 (1935)
[629] [illegible] 1409 (1940)
[630] [illegible] 1251 (1941) [*C A*
[631] [illegible]
[632] [illegible] 27, 167 (1944)
[633] [illegible] *J* 39, xvi (1945).
[634] L H Lampitt and D H F. Clayson, *Biochem J* [illegible]

does exist. Dodds[635] has shown that ascorbic oxidase is fairly specific with respect to substrate structure, and Boswell[636] has suggested that there are two systems oxidizing ascorbic acid in *Brassica napus* L., one an oxidase and the other a dehydrogenase

Dawson and his school have made very considerable advances recently in the chemistry of the oxidase They[637] conclude that the oxidase of *Cucurbita pepocondensa* is a specific copper-protein, blue-green in color, molecular weight about 150,000, with six copper atoms per molecule Their non-crystalline preparation is, on ultracentrifugal and electrophoretic grounds, homogeneous Ascorbic acid reversibly bleaches the enzyme, which is not a nucleoprotein and does not contain calcium or phosphate (calcium phosphate was a component of one of Lampitt's models) The enzyme is inhibited by cyanide, and the copper is not ionic the ionic copper associated with their enzyme may be removed on a resin without injuring the enzyme[637] The copper-protein bond appears to be non-dissociable at physiological pH's The inactivation of the enzyme during the reaction is thought to be due not to a loss of copper but to a change in the protein Copper-64 exchanges with the copper of the enzyme only during active catalysis of *the oxidation of ascorbic acid*[638] *It is suggested that during the reaction* tetracovalent-coplanar cupric copper is reduced to a less stable tetracovalent-tetrahedral cuprous copper and that this weaker structure allows exchange and possible inactivation to take place These results seem to refute the claim that ascorbic oxidase is a specific agency combined with ionic copper,[639] but Clayson and Beesley[640] claim that oxidase activity in a cucumber enzyme is independent of the copper content of the system

No corresponding animal oxidase has yet been found (cf p 425) Cytochrome c digested with pepsin behaves as an ascorbic oxidase, reducing oxygen to hydrogen peroxide and having no activity in the cytochrome oxidase or succinic oxidase systems Hydrocyanic acid, carbon monoxide, methyl isocyanide, and nitrosobenzene inhibit the oxidation[641] Gontarski[642] states that the pharyngeal gland of the honeybee secretes an ascorbic acid-oxidizing enzyme

2 Other Modes of Oxidation

Ascorbic oxidase may be the most important system oxidizing ascorbic acid in the plant, but there are very probably other systems, perhaps similar

[635] M L Dodds, *Arch Biochem* 18, 51 (1948).
[636] J G Boswell, *Ann Botany* 14, 512 (1950)
[637] M Joselow and C R Dawson, *J Biol Chem* 191, 1 (1951)
[638] M Joselow and C R Dawson, *J Biol Chem* 191, 11 (1951)
[639] A Sreenivasan and S D Wandrekar, *Nature* 165, 318 (1950).
[640] D H F Clayson and J A Beesley, *Chemistry & Industry* 70, 191 (1951)
[641] C L Tsou, *Biochem J* 49, 367 (1951)
[642] H Gontarski, *Z Naturforsch* 3b, 245 (1948)

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[625] R M Kark, R R M Croome, J Cawthorpe, D M Bell, A Bryans, R J MacBeth, R E Johnson, F C Consolazio, J L Poulin, F H L Taylor, and R C Cogswell, *J Applied Physiol* 1, 73 (1948)

[626] M Thérien and L P Dugal, *Rev can biol* 8, 248 (1949)

[627] M Thérien, J Leblanc, O Héroux, and L P Dugal, *Can. J Research* 27E, 349 (1949)

[627a] M M Monier and R J Weiss, *Proc Soc Exptl Biol Med* 80, 446 (1952)

[627b] L.-P Dugal and G Fortier, *J. Applied Physiol* 5, 143 (1952)

[627c] [illegible] 508 (1953)

[628] [illegible] 1935)

[629] [illegible] 1940)

[630] [illegible] 41) [C A

[631] F J Dunn and C R Dawson, *J Biol Chem* 189, 485 (1951)

[632] W H Powers, S Lewis, and C R Dawson, *J. Gen Physiol* 27, 167 (1944)

[633] L. H Lampitt, D. H F Clayson, and E M Barnes, *Biochem J* 39, xvi (1945).

[634] L H Lampitt and D H F. Clayson, *Biochem. J.* 39, xv (1945).

4. The Reduction of Ascorbone by Substances Other Than Thiols

The first demonstration that ascorbic acid is concerned in the oxidation of substrates other than thiols, which are not energy-providing substances in the ordinary sense, is due to James and his collaborators [412, 413] It was first shown that barley saps catalyzed in the presence of ascorbate the oxidation of lactate to pyruvate, and then that coenzyme I, as opposed to the coenzyme II of glutathione reductase, also reacted with ascorbone. The demonstration of such a system in animals has not been made In 1949 Davison[414] showed that a coenzyme I-ascorbic acid-ascorbic oxidase system partly mediated the oxidation of formate in green peas Matthews[415] examined the same system and suggested that monodehydroascorbic acid may be an intermediate, being formed by a reaction between ascorbic acid and oxygen not requiring ascorbic oxidase Ascorbone itself was ineffective in the system Tewfik and Stumpf[416] have shown that pea leaf lyophilyzates oxidize fructose diphosphate in a system requiring flavin adenine dinucleotide, adenosine triphosphate, ascorbic acid, and catalase The system is not inhibited by F′, CN′, or iodoacetamide, and therefore does not contain enolase, cytochrome oxidase, ascorbic oxidase, or triosephosphate dehydrogenase, cytochrome c, cocarboxylase, Mg, or coenzymes I and II. Glutathione had no effect, and copper in low concentrations (ca 10^{-4} M) was a total inhibitor Lactate and glycollate were also oxidized

The reaction of ascorbone with catechol has already been mentioned (p 451) It is to be hoped that in the near future the example of the plant physiologists in investigating the role of ascorbic acid in oxidative systems will be followed by the animal biochemists, perhaps particularly in the tissues of scurvy-resistant animals

Ascorbic acid has been shown by Walter and Nelson[417] to inhibit the emission of carbon dioxide by washed sweet potato slices in nitrogen. This effect of ascorbic acid was absent after cyanide or thiols had been added

5 Photosynthesis

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412 W O James and J
413 W O James, C R
414 D C Davison, Proc
415 M B Matthews, J
416 S Tewfik and P K Stumpf, J Biol Chem 193, 527 (1951)
417 E M Walter and J M Nelson, Arch Biochem 10, 375 (1946)
418 P A Kolesnikov, Doklady Akad Nauk S.S.S.R 60, 1353 (1948).

to those in animals, that can promote this oxidation. Waygood[643] has proposed that wheat seedlings contain the system coenzyme I, flavin, a pigment, and ascorbic acid· the proposed ascorbic oxidase is sensitive to carbon monoxide and presumably differs from that of Dawson. Bokuchava, Popov, and Shubert[644] have recently confirmed earlier work that ascorbic acid may be oxidized by the *o*-quinone formed in the presence of oxygen and polyphenol oxidase, and Roberts and Wood[645] found that ascorbic acid linearizes the autoxidation of catechol in the presence of a tea leaf "oxidase"

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Recently two independent teams have shown that plants contain a glutathione reductase which may well be a part of the ascorbic acid-glutathione systems carrying hydrogen from substrates to oxygen Conn and Vennesland[650] have shown that wheat germ contains a glutathione reductase highly specific for coenzyme II and glutathione, the reaction so far not having been shown to be reversible. They report that Rall and Lehninger have[384] in the same laboratory found an apparently identical enzyme in a variety of animal tissues. Mapson and Goddard[651] have independently characterized a very similar enzyme in extracts of dry pea seeds.

[643] E. R. Waygood, *Can J. Research* C,28, 7 (1950).
[644] M. A. Bokuchava, V. R Popov, and T. A. Shubert, *Doklady Akad. Nauk S.S S R.* 76, 439 (1951).
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[illegible])39)
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[illegible]
[649] V. L. Kretovich, A A. Bundel, and T. V. Drozdova, *Biokhimiya* 13, 332 (1948).
[650] E E Conn and B. Vennesland, *J Biol. Chem* 192, 17 (1951).
[651] L W. Mapson and D R Goddard, *Biochem J.* 49, 592 (1951).

4 The Reduction of Ascorbone by Substances Other Than Thiols

The first demonstration that ascorbic acid is concerned in the oxidation of substrates other than thiols, which are not energy-providing substances in the ordinary sense, is due to James and his collaborators[652, 653] It was first shown that barley saps catalyzed in the presence of ascorbate the oxidation of lactate to pyruvate, and then that coenzyme I, as opposed to the coenzyme II of glutathione reductase, also reacted with ascorbone. The demonstration of such a system in animals has not been made. In 1949 Davison[654] showed that a coenzyme I-ascorbic acid-ascorbic oxidase system partly mediated the oxidation of formate in green peas. Matthews[655] examined the same system and suggested that monodehydroascorbic acid may be an intermediate, being formed by a reaction between ascorbic acid and oxygen not requiring ascorbic oxidase Ascorbone itself was ineffective in the system Tewfik and Stumpf[656] have shown that pea leaf lyophilyzates oxidize fructose diphosphate in a system requiring flavin adenine dinucleotide, adenosine triphosphate, ascorbic acid, and catalase. The system is not inhibited by F′, CN′, or iodoacetamide, and therefore does not contain enolase, cytochrome oxidase, ascorbic oxidase, or triosephosphate dehydrogenase, cytochrome c, cocarboxylase, Mg, or coenzymes I and II Glutathione had no effect, and copper in low concentrations (ca 10^{-4} M) was a total inhibitor Lactate and glycollate were also oxidized

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643 E R Waygood, *Can J. Research* **C,28**, 7 (1950).
644 M A. Bokuchava, V R Popov, and T. A. Shubert, *Doklady Akad Nauk S S S R* 76, 439 (1951)
645 [illegible]
645a [illegible]
646 [illegible] 1939)
647 [illegible]
648 [illegible]
649 V. L Kretovich, [illegible] *nya* 13, 332 (1948).
650 E E. Conn and B Vennesland, *J Biol. Chem.* **192**, 17 (1951)
651 L. W. Mapson and D R. Goddard, *Biochem J.* **49**, 592 (1951).

in strawberries is diminished by shading the whole plant but not by shading the berries only, and Venkataramani[667] has similarly shown that glass and, to a greater extent, darkness reduce the ascorbic acid in the leaves of *T foenum-graecum* Somers and Kelly[668] have shown that the electrolytic environment of turnip and broccoli leaves influences their accumulation of ascorbic acid and dry matter under illumination 8-Hydroxyquinoline, which has a high affinity for trace metals, reduces photosynthesis and the accumulation of ascorbic acid

6 Synthesis

The connections of ascorbic acid and photosynthesis already mentioned suggest that ascorbic acid, like glucose, may be made by a photosynthetic process directly, or from glucose, its probable precursor in animals [669, 670] Prokoshev and Petrochenko[672] showed that in pieces of potato tuber after injury the content of ascorbic acid rises up to a certain level, by synthesis This is thought to result from changes in the proteins, and artificial infiltration of ascorbic acid before injury prevents the subsequent rise

The synthesis of ascorbic acid by germinating plants has been long known and made use of for dietetic purposes for many years Rangnekar, De, and Subrahmanyan[673] have shown that germination, followed by scalding to destroy the oxidase, increases the concentration of ascorbic acid in soy milk Mapson, Cruickshank, and Chen[674] have shown that in cress seedlings grown in the dark the formation of hexoses and ascorbic acid takes place at the expense of sucrose D-Mannose depressed the synthesis of ascorbic acid, whereas D-fructose, D glucose, and D-galactose promoted it, apparently by raising the hexose in the cell Noggle and Watson[675] have shown under controlled conditions a significant negative correlation between ascorbic acid and sugar in immature oat plants Miller and Jablonski[676] show that, in grapefruit, embryos contain 3 36 mg and 44 8 mg and whole seeds 2 07 mg and 31 0 mg of ascorbic acid before and after germination Carotene shows very similar post-germination to pre-germination

[667] K S Venkataramani, *Proc Indian Acad Sci* 32B, 112 (1950)
[668] G F Somers and W C Kelly, *Plant Physiol* 26, 90 (1951)
[669] B A Rubin, E V Artzichovskaja, N S Spiridonova, and O T Lutikova, *Biokhimiya* 4, 260 (1939)
[670] G A Zepkova, *Compt rend acad sci U R S S* 48, 655 (1945)
[672] S M Prokoshev and E I Petrochenko, *Doklady Akad Nauk S S S R* 61, 313 (1948)
[673] Y B Rangnekar, S S De, and V Subrahmanyan *Ann Biochem Exptl Med (India)* 8, 99 (1948)
[674] L W Mapson, E M Cruickshank, and Yu-tuan Chen, *Biochem J* 45, 171 (1949)
[675] G R Noggle and S A Watson, *Plant Physiol* 24, 265 (1949)
[676] E V Miller and J R Jablonski, *Food Research* 14, 492 (1949)

ascorbic acids, unlike some twenty other common metabolites, stimulated

tion of which resulted in products capable of oxidizing ascorbic acid or chlorophyll Tolbert, Clagett, and Burris[660] have shown that 0 0033 *M* ascorbic acid partly inhibits the oxidation of glycolic acid to glyoxylic acid by a partly purified tobacco leaf enzyme system Tolbert and Burris[661] have shown that the glycolic-oxidizing enzyme is present in the green but not in the etiolated plant or the colorless parts of plants "Activation" by exposure of the plant to light precedes chlorophyll formation The enzymes oxidizing ascorbic acid, glyoxylic, and L-lactic acid are present in the etiolated plant, and those for the first two acids are not affected by exposure to light They have confirmed Kolesnikov's[659] demonstration that chlorophyll is oxidized by the products of the oxidation of glycolic acid. Brin and Krasnovskii[662] in a series of papers have claimed that there is a reversible oxidation-reduction system between chlorophyll and ascorbic acid, arguing that under the influence of light hydrogen moves from ascorbic acid to coenzyme I and thence to chlorophyll. They discuss the relations of ascorbic acid, ascorbone, and monohydroascorbone to reduced chlorophyll and find that ascorbic acid does not stimulate photosynthesis in *Elodea*, though ascorbone causes some increase in respiration (see, e g , ref 662)

The effect of light on the ascorbic acid content of plants has been investigated during the past twenty years, partly in relation to estimations of vitamin C in plants for dietetic purposes Sideris, Young, and Chun[663] have found that the diurnal variation of ascorbic acid in the leaves of *Ananas comosus* is more closely related to the carbohydrate supply in the leaves than to diurnal intervals, and Somers, Kelly, and Hamner[664] have found that the accumulation of ascorbic acid in leaf disks depends on light and carbon dioxide Bonetti[665] has shown that in the leaves of *Pisum sativum* and *Arisarum vulgare* ascorbic acid is increased during the day and ascorbone during the night, the total being fairly constant, an observation relevant to the findings on glycolic acid and chlorophyll given above It appears that the low ascorbone-ascorbate ratio relates to photosynthesis, and the high ratio to respiration Robinson[666] has shown that ascorbic acid

[659] P A Kolesnikov, *Biokhimiya* **13**, 370 (1948)
[660] N E Tolbert, C O Clagett, and R H Burris, *J Biol Chem* **181**, 905 (1949)
[661] N E Tolbert and R H Burris, *J Biol Chem* **186**, 791 (1950)
[662] G P Brin and A A Krasnovskii, *Biokhimiya* **16**, 453 (1951)
[663] C P Sideris, H Y Young, and H H Q Chun, *Plant Physiol* **23**, 38 (1948)
[664] G F Somers, W C Kelly, and K C Hamner, *Arch Biochem* **18**, 59 (1948)
[665] D. Bonetti, *Boll soc ital biol sper.* **25**, 337 (1949)
[666] W. B. Robinson, *J. Agr Research* **78**, 257 (1949).

by biochemical and physiological tests, and experimental surgery The main results may be summarized thus In a healthy male volunteer weighing 158 lb, consumption of a diet virtually devoid of ascorbic acid led to clinical scurvy in 161 days The syndrome included a moderate loss of weight, hyperkeratosis of the hair follicles, perifollicular petechiae, extreme fatigue, and a failure to promote healing of an experimental wound. There were very slight decreases in the X-ray density of the alveolar bone. The syndrome did not include changes in the gums or teeth, acne, reduced capillary resistance, or gross subcutaneous hemorrhage It cleared up completely after intravenous injection of ascorbic acid

The estimations of ascorbic acid in the blood were of great interest. The plasma concentration fell to zero on the 41st day and remained there until therapy was begun The white-cell-platelet layer concentration fell to zero some time after the 103rd day The first clinical abnormality, follicular hyperkeratosis, was noticed on the 134th day Thus the plasma level was zero for 93 days, and the white-layer level was zero for not less than 12 days before the first evidence of scurvy was manifest These findings have been substantially confirmed by the later experiments on human ascorbic acid deficiency The M R.C Special Committee[129] found exacerbation of acne and gum changes in some subjects and used the scorbutic or nearly scorbutic subjects in an investigation of the minimum quantities of ascorbic acid needed to prevent scurvy They showed that 10 mg of ascorbic acid per day reversed or prevented scorbutic changes Pijoan and Lozner[846] had previously shown that in one subject an intake of 25 mg. per day allowed maintenance of white-layer concentration of about 26 mg per 100 g, but this result has to be interpreted in the light of the work of Lowry *et al.*, discussed on p 381, which showed that the correlation between dietary and white-layer ascorbic acid is small Van Eekelen[171] produced evidence that the rate of usage of ascorbic acid in man varies directly with the blood level Before a discussion of the human requirement of vitamin C is completed, mention should be made of the deficiencies reported by Rudolph[847] and by Rietschel and Schick[848] Strangway and Strangway[849] have produced some evidence that the African disease onyalai may be partly due to C deficiency The disease involves thrombocytopenic purpura, hemorrhagic bullae on mucous membranes, hemorrhages from the intestinal and urinary tracts, and a high mortality, 2 to 8 g of ascorbic acid may effect a cure A good account of the histology of bone in human scurvy is to be found[850]

[847] W. Rudolph, *Klin Wochschr* 19, 84 (1940).
[848] H Rietschel and H Schick, *Klin Wochschr* 18, 1285 (1940)
[849] W E Strangway and A K Strangway, *Arch Internal Med* 63, 372 (1939)
[850] E A Park, H G Guild, D Jackson, and M Bond, *Arch Disease Childhood* 10, 265 (1935)

ratios. Banerjee and Nandi[677] have shown that choline, pyridoxine, pantothenate, and inositol increase the content of ascorbic acid up to the third day of germination of *Phaseolus mungi* seeds and accelerate growth By the fifth or sixth day free ascorbic acid is absent and ascorbone is maximal. Acetone, terpineol, geraniol, menthol, camphor, veronal, and chloretone have similar promoting effects on ascorbic acid, but of these only veronal accelerates the growth rate, which may therefore be independent of the levels of ascorbic acid (cf. 7. below). Weygand and Hofmann[678] have shown considerable ascorbic acid in pollens. In summary, the mechanism of synthesis of ascorbic acid in plants is unknown, though a hexose seems to be a precursor.

7. Auxin

It seems likely from recent work that ascorbic acid is related to plant growth hormones Raadts and Soding[679] claimed that ascorbone accelerated the activation of auxin from a preformed stage, but Anker[680] has shown that indolyl-3-acetic acid (heteroauxin) promotes respiration in yeast cells, which are not thought to contain ascorbic acid, via glycogen mobilization Newcomb[681] has shown that the ascorbic oxidase activity of tobacco pith sections is strikingly increased by heteroauxin at 3 5 mg per liter of medium He suggests that ascorbic oxidase activity is thus related closely to cell growth, a relation discussed at length by Reid.[461]

VI. Deficiency and Requirement

1 Scurvy in Primates

Dr Bourne in his chapter on structural changes in vitamin deficiency has given a detailed picture of the pathological anatomy of scurvy in man and the guinea pig, and in this chapter references to scurvy in different species have been made under most of the various subheadings. The in- [illegible] [531, 683, 685] and

[illegible]

deserves very special mention as an example of how an experiment on a nutritional deficiency in a single subject should be conducted It is distinguished by repeated and painstaking medical examinations, supported

677 [illegible] *(India)* 9, 217 (1949)
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metabolized, and at a 50 mg intake about 40 mg per day was being metabolized The approach of Kellie and Zilva[693] has not been followed up, the results of their experiments, like those of Pijoan and Lozner[665] on maintenance of white-layer concentration, cannot be applied directly to the community as a whole, as very wide variations appear to exist in individual relationships between intake and saturation On the other hand, Storvick *et al* [694] have shown that 100 mg per day did not quite maintain saturation in some 18-year-old boys, Bessey and White[695] showed that in children of 5 to 13 years 45 mg per day gives maximal plasma levels of about 1 mg per 100 ml , and De and Chakravorty[696] have found that saturation in five Indian adults requires 1 0 to 2 0 mg per day per kilogram of body weight There is thus fairly general agreement that the average daily intake necessary for saturation in an adult is about 100 mg (see also pp 383 and 396)

Apart from Scheunert's work and the less easily interpreted results of Borsook *et al* ,[92] there is little evidence for the positive value of an intake allowing saturation Kyhos *et al* [697] found that the best cure of oral disease apparently due to deficiency in prisoners required a supplementation of 75 or 100 mg per day

The "theoretical" advantages of saturation are not easily deduced It is possible that kidney function is optimal when the absorption mechanism for ascorbate is saturated, but there is no evidence for this It seems that usage of C in the organism increases during infection, under certain stress conditions, and when certain substances are ingested in raised quantities, and that saturation automatically meets this usage There is no evidence that saturation is in any way disadvantageous (however, see Meiklejohn and Passmore[3]), and it can be argued that a daily dose permitting saturation should be aimed at in a conservative dietary policy A daily supplement of 100 mg of vitamin C would come to about 36 g a year, which, with L-ascorbic acid at less than a dollar an ounce in the United States, would cost a relatively trivial amount per head in that country In Great Britain this yearly amount of vitamin C costs less than one pound—a fairly small sum The experiment of supplementation is probably worth trying on a very large scale The recent report of the British Medical Association Committee on Nutrition[698] approves the figure adopted by the League of

[694] C A Storvick, B L Davey, R M Nitchals, R E Coffey, and M L Fincke, *J Nutrition* 39(1), 1 (1949)

[695] O A Bessey and R L White, *J Nutrition* 23, 195 (1942)

[696] H N De and C H Chakravorty, *Indian J Med Research* 36, 249 (1948)

[697] E D Kyhos, E S Gordon, M S Kimble, and E L Sevringhaus, *J. Nutrition* 27, 271 (1944)

[698] Report of the Committee on Nutrition, British Medical Association, London, 1950

Primates other than man are scurvy-liable, a valuable study of scurvy in the ringtail monkey has been made by Shaw.[691] Young adults took 12 to 20 weeks to show classical scurvy, with debility, moderate decreases in weight, tenderness of joints, and extensive hemorrhages in large areas in the head, arms, and legs Oral lesions were severe and progressive: there was widespread hyperemia, hypertrophy, and necrosis of gums, with extensive destruction of alveolar bone and sloughing of teeth. The early ingestion of ascorbic acid after the onset of signs led slowly to complete recovery At later stages ascorbic acid was ineffective The control group getting ascorbic acid was normal throughout. The importance of such studies in the primate are obvious, as volunteers cannot be taken as far as irreversible changes or death, and it seems that some of the classical signs of scurvy not found by Crandon, particularly the oral lesions, would have appeared if he had continued with the deficient diet. The effects of prolonged deficiency on healed wounds in monkeys would be well worth study; wounds which had healed in early deficiency (cf. ref 104) later showed degenerative changes in some of the volunteers in the M R C. experiment [128]

2 Human Requirement

The large-scale experiments on man which are needed to solve the problem of optimal requirement of vitamin C have not been conducted There seems little doubt from what has already been said that scurvy can be prevented or cured with a daily intake of some 10 mg On the other hand, Scheunert has claimed (see p 450) that supplementation of an ordinary diet with 100 mg per day leads to a lowered incidence of certain infections

The argument of Giroud and Ratsimamanga[2] from the adrenal ascorbic acid of scurvy-insensitive animals can hardly be applied to man, as there is little information for the human dietary-adrenal relation. The ascorbic acid in milk has already been discussed (p. 399), and it has been noted that the human infant of 6 months may receive 70 mg. or more per day from his mother's milk Bourne[613] has argued from observations on the large vegetarian primates, eating about 4 5 g of vitamin C per day, that human intake should perhaps keep the body saturated, and a host of experimenters have worked on the quantities of ascorbic acid needed for this purpose Kellie and Zilva,[693] from a series of measurements of plasma level and daily excretions on diets of known content, concluded that an intake of 30 mg per day allowed saturation They recommended this as a safe intake, arguing that half of this would prevent the onset of scurvy. This figure is based on work on one subject only, and the figures suggest that, as the excretion was effectively zero with an intake of 30 mg. per day, the whole intake was being metabolized At an intake of 100 mg, about 50 mg. was being

[691] J H Shaw, *Federation Proc* **8**, 396 (1949)
[693] A E Kellie and S S Zilva, *Biochem J* **33**(2), 153 (1939)

metabolized, and at a 50 mg intake about 40 mg per day was being metabolized. The approach of Kellie and Zilva[693] has not been followed up, the results of their experiments, like those of Pijoan and Lozner[695] on maintenance of white-layer concentration, cannot be applied directly to the community as a whole, as very wide variations appear to exist in individual relationships between intake and saturation. On the other hand, Storvick *et al.*[694] have shown that 100 mg. per day did not quite maintain saturation in some 18-year-old boys, Bessey and White[695] showed that in children of 5 to 13 years 45 mg. per day gives maximal plasma levels of about 1 mg. per 100 ml., and De and Chakravorty[696] have found that saturation in five Indian adults requires 1.6 to 2.0 mg. per day per kilogram of body weight. There is thus fairly general agreement that the average daily intake necessary for saturation in an adult is about 100 mg. (see also pp. 383 and 396).

Apart from Scheunert's work and the less easily interpreted results of Borsook *et al.*,[92] there is little evidence for the positive value of an intake allowing saturation. Kyhos *et al.*[697] found that the best cure of oral disease apparently due to deficiency in prisoners required a supplementation of 75 or 100 mg. per day.

The "theoretical" advantages of saturation are not easily deduced. It is possible that kidney function is optimal when the absorption mechanism for ascorbate is saturated, but there is no evidence for this. It seems that usage of C in the organism increases during infection, under certain stress conditions, and when certain substances are ingested in raised quantities, and that saturation automatically meets this usage. There is no evidence that saturation is in any way disadvantageous (however, see Meiklejohn and Passmore[3]), and it can be argued that a daily dose permitting saturation should be aimed at in a conservative dietary policy. A daily supplement of 100 mg. of vitamin C would come to about 36 g. a year, which, with L-ascorbic acid at less than a dollar an ounce in the United States, would cost a relatively trivial amount per head in that country. In Great Britain this yearly amount of vitamin C costs less than one pound—a fairly small sum. The experiment of supplementation is probably worth trying on a very large scale. The recent report of the British Medical Association Committee on Nutrition[698] approves the figure adopted by the League of

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[695] O. A. Bessey and R. L. White, *J. Nutrition* 23, 195 (1942).

[696] H. N. De and C. H. Chakravorty, *Indian J. Med. Research* 36, 249 (1948).

[697] E. D. Kyhos, E. S. Gordon, M. S. Kimble, and E. L. Sevringhaus, *J. Nutrition* 27, 271 (1944).

[698] Report of the Committee on Nutrition, British Medical Association, London, 1950.

Nations Technical Commission,[699a] namely, 30 mg., as a daily average intake sufficient to provide a good margin of safety, although its own recommended dietary allowance is 20 mg. for the normal adult. The National Research Council of the United States recommends 75 mg.—perhaps an uneasy compromise between the intake comfortably preventing scurvy (less than 30 mg.) and the dose allowing saturation (about 100 mg.).

3. Scurvy in the Guinea Pig

There are frequent references throughout this chapter to the effects of deficiency of ascorbic acid on the guinea pig, and the excellent reviews of Follis and of Wolbach and Bessey have been drawn on in the discussion of collagen formation (p. 392). The earliest histological signs of scurvy are to be found in the teeth (e.g., ref. 71). Wolbach and Bessey[143] concluded that the typical signs of scurvy in the guinea pig may be attributed to the general failure to form normal connective tissue, and this view still finds favor; there are, however, certain changes and defects in the metabolism and function of the C-deficient guinea pig that are not explicable in terms of this failure, though they and this failure may have ultimately a common cause.

The more important of these are: the failure to metabolize tyrosine (p. 431), the reduction in succinic dehydrogenase activity in the muscles (p. 443), the reduction in serum phosphatase (p. 442); the reduced response of the metarterioles to adrenaline (p. 391) and the lowered resistance to anesthesia and hemorrhage, the decrease in reduced glutathione and the increase of ascorbone in some tissues of the scorbutic animal (p. 382); and the reduction in glucose tolerance (p. 402). Lowered adrenal cortical response to corticotropin in scurvy has not been proved (p. 409).

It appears from the studies of tissue metabolism that have been possible in the guinea pig, but not in man, that the defect in collagen formation, though perhaps the most important defect in the growing animal, is caused, together with the large variety of less striking changes, by a more general metabolic defect. This defect would appear to relate to oxidation-reduction processes, a largely speculative hypothesis is that this defect springs from a shortage of the monohydroascorbone free radical, and that this radical is essential or important in a variety of special processes involving oxidations partly independent of the main stream of substrate dehydrogenation. Succinic dehydrogenase appears to be an exception (p. 431). The possible action of the ascorbone-ascorbate system as an oxygen-transporting system in the erythrocyte, the eye and, even more speculatively, in the nervous system or the lung may be independent of the free radical.

[699a] Technical Commission on Nutrition (Report on work of its third session), *League Nations Bull. Health Organisation* 7, 475 (1938).

4. Requirement in the Guinea Pig

The vitamin C requirement of the guinea pig has been fully reviewed by Mannering,[700] who gives an excellent table drawn from a large number of authors. This is condensed here as Table 11, which provides a summary of the doses of ascorbic acid needed to maintain certain functions in the guinea pig and is largely self-explanatory. It seems that a regular intake of 2 mg. per day completely prevents scurvy and that 5 mg. per day is an abundant

TABLE 11

Ascorbic Acid Requirement of the Guinea Pig[700]

Criterion of deficiency	Daily requirement,* mg
Growth	0.4
	0.6
	1– >1
	2
Macroscopic scurvy	0.5
Microscopic scurvy	1.3
	2
	2.4
	>2.5
	1†
Odontoblast growth	2
Wound healing	2
Bone regeneration	2‡
Serum phosphatase	0.23
Reproduction	2–5
Prolonged survival	5 or less
Tissue saturation	25
	50 or more

* Per animal (oral), unless otherwise indicated. Animals weighing 250 to 350 g were customarily employed.

† Per 100 g.

‡ Parenteral.

safe dose. Bruce[701] found that 5 mg. per day during growth with 20 (but not 12) mg. per day during the reproductive period allowed perfect growth and reproduction in five generations of guinea pigs. Pfander[702] has recently investigated the factors, particularly hormonal ones,[702a] affecting the requirement of the guinea pig.

Penny and Zilva[70] in a very important paper (see p. 379) have confirmed

[700] G. J. Mannering, *Vitamins and Hormones* 7, 201 (1949).

[701] H. M. Bruce, *J. Hyg.* 48, 338 (1950).

[702] W. H. Pfander, *Microfilm Abstr.* 11, 809 (1951).

[702a] W. H. Pfander, *J. Nutrition* 47, 487 (1952).

Zilva's[703] result that, as long as a guinea pig is getting 2 mg. a day before going on the deficient diet, previous saturation does not delay death. They also showed that under certain experimental conditions the onset of scurvy is not closely related to blood level. They argue that saturation in the guinea pig is in effect "supersaturation," and they use this argument in a discussion of man's requirement It is difficult to argue safely from one species to another To quote Penney and Zilva (ref. 70, p 696): "Guinea pigs, unlike human beings, do not eliminate ascorbic acid in the urine when the vitamin is consumed orally unless the doses ingested are large (300–500 mg). On the other hand the parenterally introduced vitamin is rapidly eliminated by guinea pigs after very much lower doses (25–50 mg.) . . the restricted capacity of the guinea pig for absorption of ascorbic acid is partly responsible for this difference " A single parenteral dose of 25 mg is capable of saturating the guinea pig with a weight of 300 g The proportional dose for a man of 60 kg is 5 g , which is very close to the actual dose for saturation of a scorbutic subject An oral intake of 500 mg in the guinea pig corresponds with 100 g in man. Penney and Zilva estimate by extrapolating blood values measured at intervals in guinea pigs on a scorbutogenic diet that the "true" saturated value for guinea pig blood is 0 25 mg per 100 ml , a figure about a quarter or a fifth of the figure usually accepted for man The whole animal contains 8 5 mg of ascorbic acid when "truly" saturated, the whole man contains about 5 g —some six hundred times as much as an animal of one two-hundredth his weight.

On a diet with cabbage *ad lib* the guinea pig ingests more than 25 mg per day Man absorbs nearly all his ingested ascorbic acid, and this difference alone would suggest that arguments from one species to the other, in terms of requirement, are suspect· another telling difference lies in the leucocytes Guinea pig leucocytes retain ascorbic acid (or some very similar substance) at a low steady level at the later stages of a scorbutogenic regime and develop scurvy at this steady level In Crandon[104] a zero level preceded clinical signs by at least 12 days This paper of Penney and Zilva[70] is the most important and stimulating so far published on the overall quantitative aspects of ascorbate metabolism in the guinea pig: it does not, however, really permit their conclusion about saturation in man, that "the elimination of ascorbic acid by the kidney is in all probability the extreme expression of overdosage." Ascorbic acid is normally excreted by scurvy-insensitive animals (p 399)

5 Inhibitors and Antivitamins

Woolley and Krampitz[704] found that D-glucoascorbic acid in massive amounts produced changes in the mouse which they regarded as similar to

[703] S S Zilva, *Biochem J* 30, 1419 (1936)
[704] D W Woolley and L O Krampitz, *J Exptl Med* 78, 333 (1943)

those of scurvy in the guinea pig Banerjee and Elvehjem[704a] did not agree with their interpretation that glucoascorbic acid is an antimetabolite of ascorbic acid, and Gould[704b] failed to find any antagonism to L-ascorbic acid after feeding D-glucoascorbic, 2-keto-D-gulonic, D-isoascorbic, or D-ascorbic acid to guinea pigs, using the measurement of serum phosphatase and histological tests as indicators of scurvy

Gould, Goldman, and Clarke[706] failed to find antagonism to L-ascorbic acid from 3-methyl L-ascorbic acid and 1-methylheteroascorbic acid fed to guinea pigs Shafer[654a] fed D-glucoascorbic acid to rats, hamsters, and guinea pigs Some systemic reactions, but no evidence of scurvy, were found, and D-glucoascorbic acid was shown to have some antiscorbutic action Gorlin[707] came to similar conclusions with mice The toxicity of glucoascorbic acid in large doses has been attributed to inanition secondary to diarrhea and the acidity of the diet [708] Woolley[708a] has reviewed some of this evidence, it seems clear that metabolic antagonism between glucoascorbic acid and ascorbic acid is not yet proved [708]

6 Hypervitaminosis

No striking hypervitaminotic effects have been seen with ascorbic acid such as those reported for vitamin A This may be related to the comparatively large dietary levels of vitamin C normally ingested and to its ready oxidation and excretion Effects from excess have been reported by Widenbauer,[709] who suggested that these might be due to idiosyncratic sensitivity Abt and Farmer[710] refer to vagotonic symptoms in children receiving large doses of ascorbic acid Rietschel[711] concluded that human hypervitaminosis was not of practical importance Wilson and Lubschez[712] observed that white-layer concentrations began to decrease as dietary ascorbic acid passed the level of 9 mg per kilogram per day in children aged 2 to 14 years and noted slight signs of toxicity Neuweiler[713] has claimed that, although fairly large daily supplements of synthetic ascorbic acid are without gross toxic effect on the living guinea pig, fertility and fetal survival may be severely decreased (cf ref 701) It may be that these

[704a] S Banerjee and C A Elvehjem, *Proc Soc Exptl Biol Med* **60**, 4 (1945)
[704b] B S Gould, *Arch Biochem* **19**, 1 (1948)
[706] B S Gould, H M Goldman, and J T Clarke, *Arch Biochem* **23**, 205 (1949)
[707] R J Gorlin, *J Dental Research* **29**, 208 (1950)
[708] Anonymous, *Nutrition Revs* **9**, 177 (1951)
[708a] D W Woolley, A Study of Antimetabolites, John Wiley and Sons, New York, 1952
[709] F Widenbauer, *Klin Wochschr* **15**, 1158 (1936)
[710] A F Abt and C J Farmer, *J Am Med Assoc* **111**, 1555 (1938)
[711] H Rietschel, *Klin Wochschr* **18**, 923 (1939)
[712] M G Wilson and R Lubschez, *J Clin Invest* **25**, 428 (1946)
[713] W Neuweiler, *Intern Z Vitaminforsch* **22**, 392 (1951).

effects are similar to those found after large doses of glucoascorbic acid, and they may be related to a disturbance of the normal ascorbone-ascorbate ratios in the tissues or body fluids. Recently[713a] four adults took 1 g. ascorbic acid daily by mouth for three months without ill effects or significant changes in white-layer ascorbate.

7. Toxic Substances

a. Metals. Rosenberg[1] refers to the early work on the "protective" effect of ascorbic acid against metals such as lead and arsenic. Farmer, Abt, and Aron showed[714] that arsenicals and iron tended to lower the plasma ascorbic acid, but that bismuth was without effect. Marchmont-Robinson[715] suggested that ascorbic acid promoted the excretion of lead by workers exposed to its dust, McDonald and Johnson[716] found that sensitivity to neoarsphenamine was independent of the ascorbic acid in the plasma of guinea pigs

Frommel and Beck[717] have shown that arsenic, gold, mercury, bismuth, and lead "mobilize" the ascorbic acid in guinea pig tissues and thus vitiate plasma and urine levels as indices of nutriture. Friend and Ivy[718] found ascorbic acid with hesperidin methyl cholcone to be protective in mice against poisoning with an arsenical Uzbekov[719] injected 6 mg. lead acetate per kilogram per day into guinea pigs and showed that after 50 days the levels of ascorbic acid had fallen by about 60% in brain and 80 to 85% in liver, kidney, and blood, and that the glutathione levels in the tissues had fallen by 30 to 50%. Dominicis[720] found that guinea pigs were protected against the toxic effects of manganous sulfate by ascorbic acid. Ascorbic acid can lessen stomatitis in man during treatment of syphilis with bismuth[721] and susceptibility in the guinea pig to mercury cyanide[721a] The mechanisms of the action of ascorbic acid are obscure. They may involve the kidney, the adrenal cortex, the state of oxidation of thiol groups, or the raising of tissue ascorbic acid by a mass action effect working against the tissue-depleting effects noted by Frommel and Beck[717] and by Uzbekov.[719] Possibly the conversion of ascorbate to oxalate (see p. 445) and the general

713a O H Lowry, O A Bessey, and H B Burch, *Proc Soc Exptl. Biol. Med* 80, 361 (1952)

714 C J Farmer, A F Abt, and H C S Aron, *Proc Soc Exptl Biol Med* 44, 495 (1940)

715 S W Marchmont-Robinson, *J Lab Clin Med* 26, 1478 (1941)

716 [illegible] 43, 682 (1941)

717 [illegible]

718 [illegible] 948)

719 [illegible]

720 [illegible]

721 [illegible] *tol syphilig* [illegible] 57, 515 (19[illegible])

721a M Vauthey, *Praxis* 40, 284 (1951)

insolubility of heavy metal oxalates are relevant factors Certainly the effect of heavy metals in depleting ascorbic acid in tissues is relevant to a discussion of the optimal dietary level of ascorbic acid. Its reversal of cobalt polycythemia is discussed on p 437.

b. Other Toxic Substances The effects of chloretone and other substances on rats and other animals in causing raised excretion of ascorbic acid have been discussed on p 444 There is some evidence that similar substances in man and the guinea pig cause depletion of the vitamin Meyer[722] showed that chronic benzene poisoning increases the need for ascorbic acid in man and demonstrated a simultaneous decrease in excretion of glucuronic and ascorbic acids in a human subject on withdrawing ascorbic acid. One gram of thymol caused a marked increase in excretion of glucuronic acid and a decrease in that of ascorbic acid Frommel and Beck[717] have shown that a very large variety of organic drugs, in addition to metallic compounds, decrease guinea pig tissue levels of ascorbic acid and emphasize the misleading nature of estimations of plasma levels after treatment with such drugs p-Aminosalicylic acid decreases ascorbic acid in the liver of the guinea pig and increases the scorbutic index [723] Sah[725] has shown that ascorbic acid may be compounded with bis-(4-aminophenyl)sulfone to give a substance of fairly low toxicity to guinea pigs which may be of antitubercular value Ascorbic acid increases tolerance to diethylstilbestrol,[726] to sedormid,[727] which may cause thrombocytopenia, and, in conjunction with vitamin K, to dimercaptopropanol [728]

As with the metallic poisons, the mode of action of ascorbic acid in protecting the organism against organic compounds is obscure, and probably no single mechanism is operative

It may be noted here that large doses of ascorbic acid have been used with some success in allergic conditions in man (see, e.g., ref 729)

8 Relations with Other Nutrients

a. Vitamin A. Among other workers, Mayer and Krehl[730, 731] reported that rats fed on an essentially synthetic diet lacking vitamin A showed signs of acute scurvy, with reduced ascorbic acid in blood and adrenals,

[722] A Meyer, *Vitamine und Hormone* **2**, 270 (1942) [*Nutrition Abstracts & Revs*, **17**, 922 (1948)]
[723] L. Donatelli and L Buffoni, *Boll soc ital biol sper* **25**, 1383 (1949)
[725] P P T Sah, *Rec trav chim* **69**, 277 (1950)
[726] K J Karnaky, *Surg Gynecol Obstet* **91**, 637 (1950)
[727] F Belloni, *Haematologica* **35**, 115 (1951)
[728] U Vaccaro, *Acta Vitaminol* **4**, 67 (1950)
[729] E A Brown and S L Ruskin, *Ann Allergy* **7**, 65 (1949)
[730] J Mayer and W A Krehl, *Arch Biochem* **16**, 313 (1948)
[731] J Mayer and W A Krehl, *J Nutrition* **35**, 523 (1948).

signs that could be improved by large (50 mg.) doses of ascorbic acid (see also refs 732 and 733). Mapson and Walker[734] using pair-fed controls attributed similar effects to general inanition on the vitamin A-deficient diet and, using chloretone, found no direct relation between vitamin A and the synthesis of ascorbic acid. Other recent work has been more concerned with hypervitaminosis A and scurvy. Rodahl[735] claimed that prolonged administration of excess vitamin A to various species causes a syndrome resembling scurvy in some details. Eeg-Larsen and Pihl[736] found that rats suffering from vitamin A intoxication do not show a reduced level of ascorbic acid in the serum and that ascorbic acid affords no protection against excess vitamin A, thus agreeing with Moore and Wang.[737] Maddock, Wolbach, and Maddock[738] did not state that they found signs of scurvy in a dog treated with excessive vitamin A. The catabolism of L-tyrosine appears to be depressed in guinea pigs with hypervitaminosis-A,[739] but there is no adequate ground for attributing this to a change in vitamin C metabolism

Ascorbic acid therapy has been reported to cause a significant lowering of plasma or serum carotene in children, and it may be implicated in the conversion (oxidation) of carotene to vitamin A in the gut wall

b. Other Nutrients. Apart from vitamin A, the still hypothetical "vitamin P," and the yet more hypothetical "vitamin R," ascorbic acid storage has been shown to be reduced in some pellagrins,[740] and in tryptophan deficiency in the rat[741] there results a reduction of ascorbic acid in the Harderian gland De Felice[742] has shown that excessive nicotinamide causes early death in guinea pigs fed a scorbutigenic diet, excess pyridoxine aggravates some signs in scurvy, with cyanosis of the anterior extremities, whereas choline has little effect. Biotin at a suitable dosage can increase survival in the absence of dietary ascorbic acid,[743] and there is some synergism between ascorbic acid and *meso*-inositol.[744] Mason, Casten, and Lindsay[745]

[733] C F Bassett, J K Loosli, and F Wilke, *J Nutrition* 35, 629 (1948)
[733] N B Guerrant, *Science* 108, 724 (1948)
[734] L W Mapson and S E Walker, *Brit J Nutrition* 2, 1 (1948)
[735] K Rodahl, *Nature* 164, 531 (1949)
[736] N Eeg-Larson and A Pihl, *Acta Pharmacol Toxicol* 7, 367 (1951)
[737] T Moore and Y L Wang, *Biochem J* 39, 22 (1945)
[738] C L Maddock, S B Wolbach, and S Maddock, *J Nutrition* 39, 117 (1949)
[739] B S Simić, H M Sinclair, and B B Lloyd, in press (1953).
[740] G A Goldsmith, A T Ogaard, and D F Gowe, *Am J Med Sci*, 200, 241 (1940)
[741] A Pirie, *Brit J Nutrition* 2, 14 (1948)
[742] F de Felice, *Boll soc. ital biol sper* 25, 887, 888, 889 (1949)
[743] F de Felice, *Boll soc ital biol sper* 26, 1531 (1950)
[744] F. de Felice, *Boll soc ital biol sper* 26, 1658 (1950)
[745] M F Mason, G Casten, and A. Lindsay, *Proc Soc Exptl Biol. Med* 75, 303 (1950).

have shown that ascorbic acid and *p*-aminobenzoic acid given together, but not separately, significantly increase the survival times of nephrectomized rats. It is obviously difficult to generalize about these nutrient interrelationships Some deficiencies of B-complex vitamins may be obviated in some rats by adding a few per cent of ascorbic acid to the diets[745a, 745b]

9. Deficiency in Other Animals

Miwa[746] has stated that the marmot can develop scurvy, but no other mammals apart from primates and guinea pigs appear to need dietary ascorbic acid, although McLaren *et al* [748] recommend one part in a thousand in a diet for rainbow trout fry

The work of Reid[749] suggests that in the summer young male chicks gain weight better on ascorbic acid-supplemented diets, but not in winter, whereas females grow better *with ascorbic acid in both seasons* Scurvy has not been demonstrated in birds, but Reid's findings support the view that enough ascorbic acid to prevent scurvy may not be optimal The mechanism of the growth stimulation is obscure and may involve microbiological synthesis of other factors in the gut, it has not been proved that increased growth is "beneficial," but it may be tentatively accepted, in the absence of other evidence, that under certain conditions and in some senses ascorbic acid is to the growing chick as arginine is to the growing rat

VII. Metabolism in Bacteria

Ascorbic acid differs from the other water-soluble vitamins, those of the B-complex, in its absence from nearly all microorganisms. Certain protozoa[750, 751] need the vitamin or its analogues in their food sources, and a few bacteria synthesize it The growth of some species is retarded by it (see ref 1) The almost complete microbial independence of ascorbic acid contrasts with its role in the growing multicellular plant or animal and its relation, in the latter at least, to intercellular and extracellular substances may provide a key to this difference. *for such substances are of little, if any,* importance to unicellular organisms The leucocytes, free cells of animals, do, however, contain large concentrations of ascorbic acid (see p 386)

The metabolism of ascorbic acid in microorganisms has aroused some interest, since the characterization of vitamin B_{12} A review of ascorbic acid and other vitamins as nutrients for microorganisms has been written

745a F S Daft and K Schwarz, *Federation Proc* **11**, 200 (1952)
745b E G McDaniel and F S Daft, *Federation Proc* **11**, 450 (1952)
746 A Miwa, *Oriental J Diseases Infants* **26**, 3 (1939)
748 B A McLaren, E Keller, D J O'Donnell, and C A Elvehjem, *Trans Wisconsin Acad Sci* **40**, I, 259 (1951)
749 M E Reid, *Federation Proc.* **9**, 368 (1950).
750 M Lwoff, *Comp rend soc biol* **130**, 406 (1939)
751 R Cailleau, *Comp rend soc biol* **138**, 319 (1939).

by Schormuller.[752] Shive, Ravel, and Eakin[753] showed that certain lactic acid organisms apparently can dispense with vitamin B_{12} if given ascorbic acid. Welch and Wilson[755] showed that ascorbic acid augments a casein digest in promoting the growth of *Lactobacillus leichmannii* and probably reactivates vitamin B_{12}, inactivated by autoclaving at pH 10, by reduction of oxidation products. Kocher[756] showed that *Lactobacillus lactis* dispenses with vitamin B_{12} in the presence of ascorbic acid in the presence or absence of oxygen (see also ref. 475).

McNutt and Snell[758] have shown that pyridoxamine phosphate, plus a reducing agent (ascorbic acid, cysteine, or glutathione), or vitamin B_{12}, or thymidine, can replace certain natural materials essential for the growth of certain lactic acid bacteria on otherwise synthetic media. Ascorbic acid here appears to be acting as a non-specific reducing agent, as it does in promoting the production of streptolysin O by a Group A hemolytic *Streptococcus*.[759] Its effect on the metabolism of *Pseudomonas aeruginosa* has been investigated by Avezzú.[760]

Certain microorganisms reduce ascorbone. Tkachenko[761] showed this property in *Lactobacillus bulgaricus* and *Lactobacillus leichmannii*, and Goljanickij and Belosonov[762] claimed that ascorbone was reduced during alcoholic fermentation. Mapson and Ingram[763] have used the reduction of ascorbone to ascorbic acid by *Escherichia coli* in the estimation of ascorbone. This reduction is quicker than that of other oxidized ene-diols, including D-isoascorbone.

Eddy[764, 765] has shown that this reduction by *Escherichia coli* may involve two mechanisms, one of which may use glucose, and the other a variety of ultimate substrates. Cell-free preparations did not reduce ascorbone, and the missing factor or factors could not be added. This process may well not be a part of the normal physiology of *Escherichia coli*.

VIII. Summary

The importance of ascorbic acid is demonstrated by the existence of scurvy, which is found in man and a few other species. One of the mole-

752 J. Schormüller, *Z. Lebensm. Untersuch. u. Forsch.* 88, 408 (1948).
753 W. Shive, J. M. Ravel, and R. E. Eakin, *J. Am. Chem. Soc.* 70, 2614 (1948).
755 A. D. Welch and M. F. Wilson, *Arch. Biochem.* 22, 486 (1949).
756 V. Kocher, *Intern. Z. Vitaminforsch.* 20(4), 369 (1949).
758 W. S. McNutt and E. E. Snell, *J. Biol. Chem.* 182, 557 (1950).
759 H. D. Slade and G. A. Knox, *J. Bact.* 60, 301 (1950).
760 G. Avezzú, *Boll. soc. ital. biol. sper.* 27, 105 (1951).
761 E. S. Tkachenko, *Biokhimiya* 1, 579 (1936).
762 I. A. Goljanickij and I. S. Belosonov, *Compt. rend. acad. sci. U.R.S.S.* 4, 15 (1936).
763 L. W. Mapson and M. Ingram, *Biochem. J.* 48, 551 (1951).
764 B. P. Eddy, *Biochem. J.* 50, 601 (1952).
765 B. P. Eddy, M. Ingram, and L. W. Mapson, *Biochem. J.* [illegible]

cular roles of ascorbic acid now seems established It is a factor in the enzymic introduction of a second hydroxyl group into *p*-hydroxyphenylpyruvic acid formed by oxidative deamination of tyrosine The relation of this activity to the prevention of scurvy is obscure Undoubtedly ascorbic acid is essential in the production of collagen in scurvy-liable animals, and it is very likely that it is more or less specifically concerned in the oxidation of a precursor to give the adrenal cortical steroid resembling cortisone or compound F.

The other possible roles for ascorbic acid are in the metabolism of folic acid and cobalt, in the activity of the corpus luteum, in the action or inaction of hyaluronidase, in the action of adrenaline on small blood vessels, and in the transport of oxygen across membranes for special purposes The last decade has seen a shift of interest from ascorbic acid as a nutrient to ascorbic acid as a metabolite with hormonal and enzymic relationships, and from ascorbic acid as a reducing agent to ascorbone or a free radical as an oxidizing agent The synthesis of ascorbic acid in the plant and animal remains obscure Its destruction very probably entails oxidation followed by fission of the carbon chain, oxalate being a partial product The problem of human requirements and recommended allowances remains unsolved, 10 mg daily prevents scurvy, yet it is possible that ten times this intake will ensure greater freedom from minor infections and illnesses

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* Numbers in parentheses are reference numbers and are included to assist the reader when an author's name is not mentioned on the page

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